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PLASTIC EMBEDDING OF THICK CELLOIDIN SECTIONS OF NERVE TISSUE

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ABSTRACT.—**A method is described for mounting Golgi-impregnated and Weigert-stained thick celloidin sections of brain and spinal cord in transparent plastic. Finished mounts have good optical properties and are suitable for macroscopic and microscopic observation. The durability of such preparations makes them superior to similar material prepared by the more conventional methods. Holes of suitable size were cut in matrices of $2.5 \times 5 \times \frac{3}{16}$ inches Plexiglas. Ward's Bio-plastic was used to form a base for the holes and also as the embedding medium for the sections. Plate glass formed a working substrate and gave a polished surface to the plastic base and later to the top of the preparation. For Golgi material (200μ) the celloidin was removed by dioxane. A dioxane-plastic bath preceded plastic embedding. For Weigert material (30–40μ) celloidin was not removed due to fragility of sections. Prior to plastic embedding, they were subjected first to benzol and then to a benzol-plastic bath.**

The superb preparations of anatomical specimens in transparent plastic demonstrated by Kampmeier and Haviland at the A.A.A.S. meetings at Chicago in 1947, followed by publication of their methods and a good review of the field (1948), have been a stimulus to further work along these lines. In addition to mounts of gross specimens, these workers have briefly described mounting of large thick celloidin sections. In this paper we describe methods which have given us the best results with thick celloidin sections of cat and rabbit brain and spinal cord following the Golgi and Weigert technics. Completed mounts are durable, have good optical properties and may be observed with the 16 mm. lens of the compound microscope. The Golgi preparations are superior to those mounted by the balsam method.

By our method a sheet of Plexiglas of 3/16 inch thickness is sawed into rectangles 2½ by 5 inches, and two smooth-sided 2-inch holes are machined into each rectangle. This diameter easily accommodates transverse sections of cat brain. It is advisable to use a hole with a diameter somewhat larger than the specimen, in order to decrease curvature formed by a meniscus when plastic is poured. The size may be altered to fit other materials.

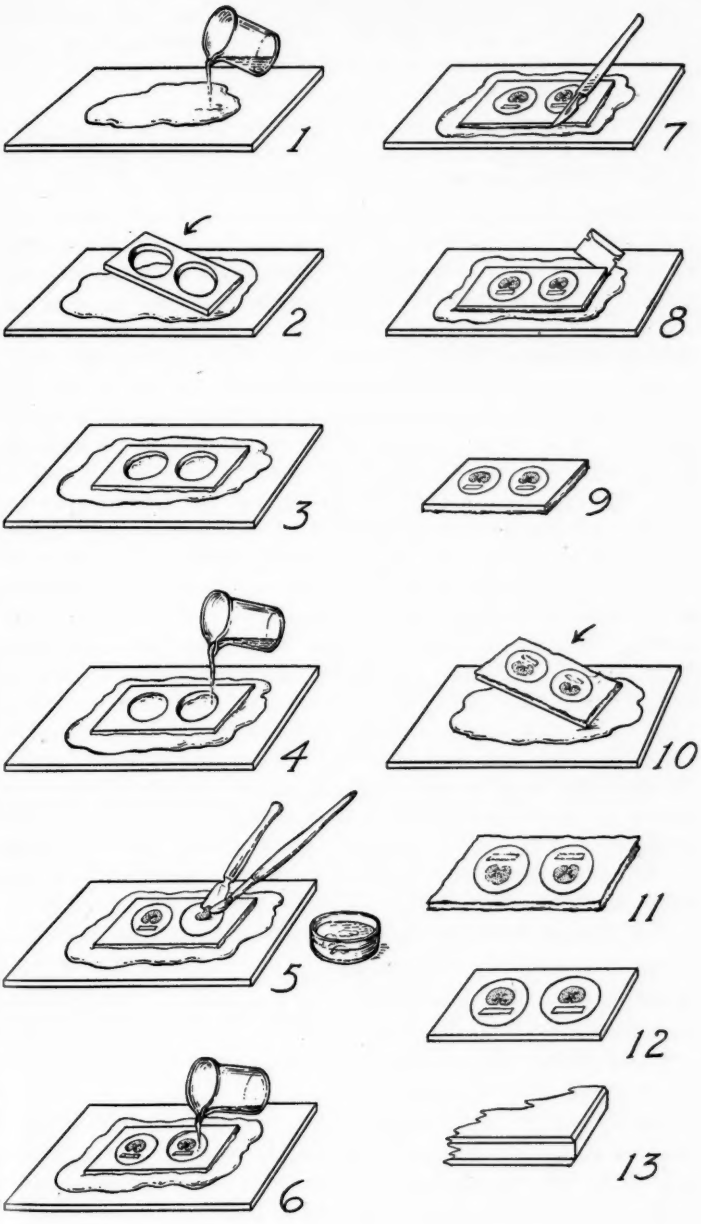
A pool of Ward's Bio-plastic plus 18 to 20 drops of catalyst per 100 ml. of plastic is poured onto a piece of clean plate glass (Fig. 1). The cell is placed upon it (Fig. 2,3), and a 24-hour room temperature hardening period is allowed. The cell is then filled with more plastic to the level desired for the section (Fig. 4). After pouring the additional plastic into the cell, the preparation is placed in a dust-free, level place to harden, first for 24 hours at room temperature, then 36 hours at 37°C., and finally at 55°C. for 12–16 hours. It is cooled slowly. The rectangles usually free themselves spontaneously from the glass base leaving a polished surface, which eventually becomes the bottom of the finished preparation. Too rapid cooling may cause the glass to shatter. In our experience plate glass is superior to ordinary window glass as a base, since the plastic frees itself more easily from it with no shattering of the glass. If the plastic does not spontaneously pull away when thoroughly cooled, the seal may be broken by lightly scoring the edge of the cell several times with a sharp, sturdy scalpel (Fig. 7) and then slightly lifting the corner with a sharp razor blade (Fig. 8).

By this procedure, excess plastic from around the edges of the mount will be left on the glass. Having been exposed to air during the curing period it will not be completely polymerized. It may be removed from the glass by gentle scrubbing with dioxane-soaked cotton. Throughly hardened plastic polymerized in the absence of air is little affected by dioxane, however, and must be carefully scraped off with a sharp razor blade.

PLATE I

A pictorial description of a method for mounting thick celloidin sections in plastic. Curing (polymerization) of the plastic occurs between Fig. 3 and 4, 4 and 5, 6 and 7 and between 10 and 11.

1–4 Preparation of a plastic base for pre-cut Plexiglas cells, using plate glass as a working substrate.
5 Floating sections onto the prepared base.
6 Pouring an additional thin layer of plastic to cover the sections and fill the cell.
7–9 Removing preparation from the glass.
10 Inversion onto new pool of plastic to finish the top surface.
11 Rough-edged preparation ready for finishing.
12 Excess plastic has been removed by band-sawing or sanding.
13 Beveling of the edges is not necessary but adds to the appearance of the preparation.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Golgi-stained Material

An outline for the procedure for material stained by the Golgi technic is as follows:

1. Remove celloidin-embedded blocks from chloroform hardener, and section at 200 μ on the sliding microtome, keeping the block and the knife flooded with 80% alcohol.

2. Transfer sections, as cut, to 95% ethyl alcohol, in which they may remain for not more than five minutes. Cut only a few sections at one time, in order that the process may continue to the next step without undue prolongation in the 95% alcohol.

3. Place the sections in equal parts 95% ethyl alcohol and dioxane until the celloidin is dissolved.

4. Transfer to pure dioxane for two minutes.

5. Use a second pure dioxane for two to five minutes to insure complete dehydration. (Slight traces of water carried into the plastic will cloud the mount badly.)

6. Place in a mixture of one-part dioxane and two parts of the uncatalyzed plastic, until the sections become well-cleared. Use an air-tight bottle, either a screw cap or rubber-sealed jar, since the mixture of dioxane and plastic (or benzol-plastic mixtures) harden in a few hours at room temperature if exposed to air. Furthermore, sections become opaque if allowed to stand in an exposed mixture. Storing in the refrigerator at 5° to 7°C. prolongs the time the mixture will remain fluid. This phase of the procedure is of practical importance, since Golgi-stained specimens to be mounted in balsam or clarite must be carried to completion in the shortest time possible. By the plastic method the tissues may be run up to the dioxane and plastic as rapidly as possible and may then be stored for future mounting. If the plastic begins to set, however, failure will ensue; no solvent has been found by which it can be reconverted to a solution. Benzol-plastic mixture will give the same results if left exposed.

7. To mount the section, float it onto a spatula and carefully slide it into the previously-hardened base in the cell (Fig. 5). (Printed labels which have been soaked for ten minutes in a plastic-dioxane mixture may also be put into the cell at this time.) Fill the cell with catalyzed plastic (Fig. 6), and set it in a *dust-free*, level place to cure for 24 hours at room temperature.

8. To finish the preparation, pour a pool of plastic plus catalyst onto the surface of the glass, as in Fig. 1, and lower the unfinished side of the cell onto the mixture (Fig. 10). Allow sufficient plastic to flow out from the edges of the cell so that they are well sealed.

Finishing time can be sped up to overnight at room temperature followed by 24 hours at 37°C. and finally 12 hours at 55°C.

Weigert-stained Material

For the Weigert stain, material is sectioned at 30 to 40μ. Benzol is used as the final bath prior to plastic, since benzol does not dissolve celloidin. This is necessary with such thinner sections which cannot be handled without a celloidin matrix. Subsequent to staining, sections are run up to 95% alcohol, then into carbo-xylol to complete dehydration and for clearing. Sections left in carbo-xylol for 5–10 minutes harden somewhat and can be handled more easily. They are placed in pure benzol for 5–10 minutes to wash out the carbo-xylol. They can remain in the benzol overnight without damage. The depression in the prepared base is flooded with benzol, and the section is transferred quickly by means of a spatula and a soft brush (Fig. 5). A pipette filled with benzol can be used to keep the spatula flooded. Excess benzol is removed from the depression with a fine pipette, and plastic is carefully brushed onto the section to prevent drying. The cell is then completely filled with plastic and the curing process as described for the Golgi material is followed.

Rough edges of the preparation (Fig. 11) are dressed by using a band-saw to remove excess plastic. The edge is smoothed (Fig. 12) with a fine file. Addition of a slight bevel (Fig. 13) to the edge improves the appearance of the specimen. It is unnecessary to polish the sides, since the specimen is not viewed from these surfaces.

Discussion

It cannot be said from our results that plastic is applicable to general histological technic, since it has here been used for silver-impregnated (Golgi) and hematoxylin-stained (Weigert) specimens only. In both cases the method has been limited to thick celloidin sections. Attempts to shorten the method by combining the steps of embedding and surfacing into a single operation proved to be unsatisfactory.

REFERENCE

Kampmeier, O. F., and Haviland, T. N. 1948. On the mounting of anatomical specimens in transparent plastics. Anat. Rec., **100**, 201–31.

ACID THIONIN STAIN FOR NISSL BODIES ON FROZEN SECTIONS

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ABSTRACT.—**Using a buffered acid thionin stain with carbol-xylene as a clearing agent, a reliable stain for Nissl bodies may be performed on frozen sections of fresh or old formalin-fixed material in a relatively short period of time. The technic is simple: the buffering of thionin makes regressive differentiation unnecessary.**

A reliable, accelerated stain for Nissl bodies in frozen sections of fresh or aged formalin-fixed tissues has been evolved. The use of buffered thionin was derived almost directly from our current technic for the staining of Nissl Bodies in paraffin sections (1) which in turn resembled closely Windle's buffered thionin (2).

To find the optimum pH level, sections from human and animal tissues were stained at different pH levels. At pH 3.5, the immediate results were satisfactory but the stains faded after a few days exposure to light. The sections stained at pH 5 were too dense for immediate examination. At pH 4 the Nissl bodies remained distinct with a slight fading of glial cell nuclei after 80 days exposure to a 60 Watt light bulb at about 20–50 cm. distance.

The stock solution of 0.1% thionin in aqueous M/100 acetate buffer of pH 4 has been kept at room temperatures of 22–35°C. for 1 year without impairment of staining qualities or appreciable shift in pH level. The solution was kept tightly stoppered and the small quantities required for use were poured out, avoiding insertion of pipettes. Acetate buffer was used because it was found that sodium acid phosphate precipitated thionin.

Heretofore, the use of carbol-xylene after basic aniline dyes has been avoided because of its fading properties. In this method 1:3 carbol-xylene extracts the stain slightly, and with a 1:4 proportion and a few seconds immersion of the section, only a negligible amount of stain is removed. In a few cases of overstaining longer immersion will remove the excess of thionin.

The sections cleared in Weigert's 1:3 carbol-xylene and control sections cleared in pure xylene were exposed to a 60 W. light bulb for 80 days. The Nissl bodies remained distinct in most instances, with slight fading of glial cell nuclei. Clearing in carbol-xylene renders the sections pliable, flat and free of wrinkles, and facilitates

mounting. After xylene clearing, sections are brittle, wavy and wrinkled, and mounting is difficult.

Three mounting media were tested for stain permanence after clearing with pure xylene and with carbol-xylene. After 80 days exposure to light, sections mounted in polystyrene and in Hartman Leddon's No. 7885 resin, with a few exceptions, presented sharply stained tigroid. With Canada balsam as the mounting medium, the sections cleared in pure xylene remained fairly satisfactory, but all of those mounted in Canada balsam after clearing in carbol-xylene faded in 1–2 days.

The Technic

Stock thionin solution:

Thionin (CC) (C.I.No. 920)	400 mg.
M/5 acetic acid solution	16 ml.
M/5 sodium acetate solution	4 ml.
Distilled water	380 ml.

Use tissues fixed in aqueous 10% formalin. Place in watch glasses or small Stender jars with covers.

1. Freeze and cut sections 10–15 μ.
2. Transfer to distilled water.
3. Transfer to 70% ethyl alcohol 5 min. (old formalin material especially).
4. Wash in distilled water.
5. Stain 10 seconds in 1–2 ml. of the 0.1% aqueous thionin in pH 4 acetate buffer.
6. Wash in distilled water.
7. Dehydrate in 95% and 100% ethyl alcohol or 2 changes of acetone.
8. Clear for few seconds in 1:4 carbol-xylene.
9. Place on slide, blot, mount in polystyrene or Hartman Leddon's No. 7885 resin.

Results

Deep blue Nissl bodies and glial nuclei on pale or colorless background with both fresh and old formalin-fixed material. If there is too blue a background, this can be corrected by exposure for a few hours to sunlight or a lamp bulb.

Acknowledgment is made to Dr. R. D. Lillie for advice and criticism.

REFERENCES

Lillie, R. D. 1948. Histopathologic Technic. Blakiston, Philadelphia, Pa.

Lillie, R. D. 1949. Histochemical studies with malt diastase. (In press)

Windle, Rhines and Rankin. 1943. A Nissl method using buffered- thionin. Stain Techn., 18, 77.

A NEW FIXATIVE FOR VAGINAL SMEARS

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ABSTRACT.—**Two vaginal smear fixatives have been presented for use in cytologic studies by the Papanicolaou technic for the diagnosis of cancer of the genital tract. They are to be used in lieu of equal parts of ethyl alcohol and ether, because of the volatility, waste through evaporation, fire hazard, and expense. They are (a) tertiary butyl alcohol, 75% and ethyl alcohol, 25% (b) tertiary butyl alcohol, 75% and ethyl phosphate, 25% (by volume). The cytologic details and staining qualities of vaginal smears have been maintained or improved.**

Since the introduction of the Papanicolaou technic of vaginal smears for ovarian endocrine studies (1933) and the cytologic detection of cancer (1945), the fixative of choice has been equal parts of ethyl alcohol and ethyl ether.

There have been several disadvantages to this mixture, namely, the expense of the ethyl alcohol to smaller laboratories and physicians who cannot obtain it tax free; the marked volatility of the mixture which during the summer months and in warm climates makes for waste and causes ether fumes (the boiling point of ether being 34.6°C.); the handling of the ether during the preparation of the mixture produces the possibility of an explosive atmosphere, especially if the laboratory is not well ventilated.

For these reasons a new fixative was sought. A fixative that would preserve the cytologic details, that would not produce shrinkage of the cells or alteration of the staining qualities; and that would be more stable, less expensive, and easily available.

Johansen (1935) first introduced tertiary butyl alcohol as a dehydrating agent for tissues. During the recent war, because of priorities on ethyl alcohol for civilian use, Stowell (1942a and b) reintroduced it in michrotechnic. He found it unusually satisfactory for the dehydration of mammalian tissues. He states "it causes less shrinkage than dioxane, xylene, or chloroform". The cytologic details are well preserved.

Tertiary butyl alcohol [$(CH_3)_3$ COH], has a molecular weight of 74.12. Below 25.5°C., the melting point, it appears as rhombic prisms

or plates. Above this temperature it is a clear, colorless liquid with a slight odor. The boiling point is 82.8°C., the index of refraction is 1.38779, the density 0.7887 at 20°C. It is soluble in water, ethyl alcohol and ether in all proportions.

Stowell (1942, a) uses tertiary butyl alcohol in conjunction with ethyl alcohol—a 100% solution containing 75ml. tertiary butyl alcohol and 25 ml. of absolute ethyl alcohol. This combination is an excellent fixative for vaginal smears. It has been used in this laboratory for the past year with gratifying results. Excellent cytological detail is maintained with little or no shrinkage. The staining characteristics using the Papanicolaou stain are maintained or slightly improved.

To completely eliminate the ethyl alcohol, a new fixative and dehydrating agent was substituted, namely, triethyl phosphate $(C_2H_5O)_3\ PO_4$. This reagent was introduced by Nelson (1945). It displaces water in tissues with slight if any shrinkage or distortion.

The physical properties of triethyl phosphate (commonly called ethyl phosphate) are, molecular weight 182.16; boiling point 218°C. It is a clear, colorless liquid, with a very slight pleasantly sweet odor. It is soluble in water, ether, ethyl and tertiary butyl alcohols and xylene. Index of refraction is 1.40616 at 10°C. Density is 1.0686 g./ml. at 25°C.

For the past year, the combination of tertiary butyl alcohol, 75%, and ethyl phosphate, 25%, has been used extensively in this laboratory for vaginal smear preparations with excellent results.

When slides are removed from this fixative it is not necessary to work rapidly to prevent the slide from drying, as there is sufficient ethyl phosphate covering the smear after the tertiary butyl alcohol evaporates, due to the high boiling point of the ethyl phosphate.

The smears are fixed in this fixative within 5 minutes and they have been left in the fixative for over 3 months without alteration in the cytologic details or staining qualities.

REFERENCES

PAPANICOLAOU, G. N. 1933. The sexual cycle in the human female as revealed by vaginal smears. Amer. J. Anat., (Supp.), **52**, 519.

PAPANICOLAOU, G. N., and TRAUT, H. E. 1945. Diagnosis of Uterine Cancer by the Vaginal Smear. The Commonwealth Fund, New York.

JOHANSEN, D. A. 1935. Dehydration and infiltration. Science, **82**, 253.

STOWELL, R. E. 1942, a. Tertiary butyl alcohol as a microtechnical reagent. J. Tech. Methods, **22**, 71.

STOWELL, R. E. 1942, b. The use of tertiary butyl alcohol in microtechnique. Science, **96**, 165.

NELSON, O. E. 1945. The use of dimethoxytetraethyleneglycol and triethylphosphate in histological technic. Stain. Techn., **20**, 131.

A CHEMICAL AND HISTOCHEMICAL STUDY OF THE TECHNIC FOR ACID PHOSPHATASE[1]

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ABSTRACT.—**A chemical and histochemical study of Gomori's acid phosphatase technic showed that the causes of its unreliability were: (1) that fixation and other steps of histological procedure inactivate the enzyme to a great extent; (2) the enzyme may diffuse, as demonstrated in frozen sections of acetone-fixed material; and (3) some absorption of lead by the sections takes place. Much of this unreliability is avoided, however, by maintaining as low a temperature as possible during fixation and dehydration, with exposure to the temperature of the paraffin oven for the shortest possible length of time. The relative insolubility and thermo-stability of the enzyme, moreover, indicate the possibility of devising a more satisfactory technic in the future.**

Ever since 1941, when Gomori described the cytochemical technic for acid phosphatase, a number of reports have appeared in the literature bringing evidence against the specificity and for the unreliability of the method. These papers have pointed out, among other things, the lack of correspondence between chemical data and cytochemical results (Heinzen, 1947), the resistance of the enzyme to inactivation, the non-specific reaction with ascorbic acid (Lassek, 1947), the reaction in injured nerves after probable destruction of enzymatic activity, and the extraction of the enzyme by water, judged cytochemically (Bartelmez and Bensley, 1947). All this work was done on nerve tissue. Stafford and Atkinson (1948) reported chemical data on the destruction of the enzyme by alcohol and acetone fixation and by paraffin embedding.

The authors have made an attempt to study the factors involved in the unreliability and in the so-called non-specificity of the method. It seemed that the unreliability could be the result of two phenomena that can take place during the histological procedure, to wit, inactivation of the enzyme and its extraction from the tissue.

The so-called non-specific reaction could be attributed to incomplete inactivation of the enzyme or to adsorption of lead on histologi-

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cal structures or both. It is interesting to note that practically all reports on false acid phosphatase reactions are on nerve tissue which has an unusual affinity for heavy metals.

We wish to report here results on the action of fixatives, histological procedures and various agents upon acid phosphatase activity, as judged by chemical and cytochemical methods.

Material and Methods

Adult rat and mouse liver was used in this investigation. Liver tissue was chosen because of its high enzyme content and the structural homogeneity of the organ. Animals of both sexes were used, since sex does not influence the enzyme content of the liver (Kochakian, 1947). The animals were killed by a blow on the head, the abdominal cavity opened, fragments of the liver removed and weighed on a torsion balance. Preliminary results showed marked uniformity in the enzyme content of 80 to 120 mg. fragments from 5 different portions of the liver.

Chemical method. Acid phosphatase activity was determined in aqueous extracts (20 to 40 mg. of tissue per ml.) made according to Greenstein (1942). When homogenates were used, 0.5 ml. of a 10% Potter-Elvehjem homogenate was blown into 10 ml. of chilled fixative. It was usually kept in the ice box for 3 hours, then centrifuged, and the precipitate, re-homogenized in saline, used for assay. If the tissue had been previously subjected to the action of fixatives or to histological procedures, enough water was added to make up to their initial weight. The King-Armstrong method as described by Gutman and Gutman (1940) was used. In the majority of the cases incubation time was one hour but when the main interest was the demonstration of the presence of small amounts of enzyme, the incubation period was extended to 12 hours. The effect of dilution of the extract on its activity showed no linear proportionality when activities of 1:2, 1:5, 1:10, 1:20 and 1:50 dilutions were determined. The activity was slightly higher than expected in the higher dilutions. Therefore, the percentages referred to in this paper are only approximate. This is to be borne in mind especially in the case of considerable inactivation in tissue blocks subjected to histological procedures.

Cytochemical method. Frozen sections were used in view of the constant results obtained by the acid phosphatase technic when applied to frozen material. Before sectioning, the blocks were kept in water for 10 minutes. Frozen sections 20 to 40μ thick were incubated in the following mixture, cleared by filtration before use: dis-

tilled water, 10 vol.; N/10 acetate buffer pH 5, 6 vol.; 2% sodium glycerophosphate (50% alpha, May & Baker), 2 vol.; and 2% lead nitrate, 2 vol. Sections were rinsed in distilled water for 2 to 5 minutes, transferred to a dilute solution of ammonium sulfide, rinsed in water, dehydrated and mounted. Controls were run in a mixture containing no glycerophosphate, and also in the complete incubating mixture to which sodium fluoride was added in a final concentration of N/400.

Results

Effect of fixatives on acid phosphatase. Chemical results obtained with fixed liver blocks are summarized in Table 1.

With homogenates less inactivation was obtained, but even so, fixation in chilled absolute alcohol for 3 hours inactivated 50% of the enzyme, while chilled acetone inactivated about 35%. The effect of dilute chilled alcohol was also studied on homogenates because of the possibility that low concentrations of fixative prevailing during the stage of its gradual penetration into the tissue may be especially harmful. However, homogenates fixed in concentrations of 17, 25, 40, 60 and 100% alcohol were found to be inactivated to about the same extent. On the other hand, the temperature of the fixative seems to be very important, for homogenates fixed at room temperature either in alcohol or in acetone showed an inactivation in excess of 80%.

Phosphatase activity was examined histochemically in 30μ thick frozen sections of liver blocks fixed in the same fixatives and for the same length of time. Incubation time was 45 and 90 minutes. Alcohol-formol-acetic-acid fixative, alcohol-formol (10:1), sublimate-alcohol and methyl alcohol completely inactivated the enzyme the, color development being equal to that of fluoride control sections. Formol-saline and 80% alcohol resulted in diminished activity as compared with acetone fixation.

Experiments were also made with frozen-dried blocks of liver in the hope that dry dehydration of the tissue would prevent inactivation of the enzyme.

Frozen-dried blocks were fixed overnight at 5°C. in 60, 80 and 100% alcohol and acetone and in formol-alcohol 1:10. They were subsequently treated in the refrigerator with 100% alcohol for 24 hours, alcohol-ether, 2 changes, one hour each, 4% collodion in alcohol-ether overnight, chloroform, 2 changes, one hour each, and 3 hours in paraffin in a 60°C. oven. Sections of these blocks did not stain darker or more uniformly than those of control blocks fixed freshly in chilled acetone.

The effect of the dehydrating and embedding procedure on acid phosphatase. Small blocks fixed for 2 and 24 hours in absolute acetone at 5°C. and subsequently carried through part or all of the embedding routine were used. The experiment is summarized in Table 2. Data on phosphatase activity are averages of 4 blocks each.

The inactivation produced by alcohol at room temperature is so great that it was thought it may overshadow other inactivating agents used later on in the routine. However, the harmful effect of neutral hydrocarbon solvent at the temperature of the paraffin oven could be demonstrated by fixing homogenates in chilled acetone, treating them for 3 hours with xylene at 60°C., washing them with acetone

TABLE 1.—EFFECT OF FIXATIVES ON THE ACID PHOSPHATASE ACTIVITY OF LIVER BLOCKS

Fixative	Time	Residual activity
	(Hours)	%
Acetone (6° C.)	24	22.0
Acetone (6° C.)	2	39.0
10% formol-saline (6° C.)	12	9.1
80% alcohol (6° C.)	12	11.4
1 part saturated aqueous $HgCl_2$ and 1 part absolute alcohol (6° C.)	12	4.0
Alcohol-formol-acetic acid (8:1:1) (6° C.)	12	1.5
Methyl alcohol (6° C.)	12	0.0

TABLE 2.—EFFECT OF DEHYDRATING AND EMBEDDING ON THE ACID PHOSPHATASE CONTENT

Acetone	100% alcohol*	Xylene*	Paraffin 56° C.	Xylene*	100% alcohol*	70% alcohol*	Water*	Residual activity
Hours	Hours	Hours	Hours	Hours	Hours	Hours	Minutes	%
24	—	—	—	—	—	—	—	28
24	2	—	—	—	—	—	—	4
24	2	2	—	—	—	—	—	2
24	2	2	3	—	—	—	—	3
2	2	3	1	1	1	1	30	7
2	—	—	—	—	—	—	—	39

*Room temperature.

and resuspending them in water. Acid phosphatase activity of the heated homogenate was only 40% of that of the unheated control.

Histochemical experiments were performed with small blocks of acetone-fixed material. They were divided in groups and treated as shown in Table 2. Frozen sections of the central portions of 2 blocks of each group were incubated for 45 min. in the substrate. Pronounced inhibition was noted in sections of blocks treated with alcohol, xylene and paraffin as compared with untreated ones.

Extraction of enzyme from tissue by fixatives and by water. It was attempted to demonstrate enzymatic activity in acetone, 80% alcohol and 10% formol-saline, used as fixatives for blocks of tissue weighing about 1 g. Time of fixation was 12 and 24 hours. The volume of the fixatives was reduced to about 6 ml. in a calcium chloride vacuum desiccator, and the phosphatase content was determined, an incubation period of 12 hours being used. The possibility that enzymatic activity in the fixatives may have derived from hemolyzed red blood cells led to the use of saline-perfused livers. This experiment showed that the phosphatase content of the liver tissue itself was not modified by this procedure. No significant activity could be detected in the fixatives. To test the possibility of the extraction of acid phosphatase from sections, fixed small blocks of about 1 g. of liver were cut in 50μ thick frozen sections. The sections were kept in distilled water at 37°C. for 4 to 5 hours. After filtration through gauze, the volume of water was reduced to 6 ml., and the activity of 2 aliquots determined. Amounts representing at most 5% of the total activity of the fixed blocks could be found. No attempt was made to recover the enzyme quantitatively from the sections.

Experiments were also made with homogenates in order to determine the solubility of acid phosphatase after fixation. A certain amount of homogenate fixed in chilled absolute acetone was resuspended in water, rehomogenized and centrifuged at 2000 r.p.m. for 10 minutes. Determinations of acid phosphatase activity were made on the total homogenate, on the washed precipitate and on the supernatant fluid. About 50% of the activity was found in the precipitate and the other 50% in the supernatant fluid.

Frozen sections of tissue fixed in acetone, 80% alcohol and 10% formol-saline were kept in water at 37°C. for 2 to 5 hours. They were then incubated in the substrate and the activity was compared with that of untreated sections. A slight decrease in activity was noted when extraction was maintained for 5 hours. No decrease in activity was seen after 1 or 2 hours.

Effect of temperature on acid phosphatase activity. A few chemical experiments were performed on aqueous extracts of liver. It could be shown that at 61°C. enzymatic activity was about the same as at 37°C. At 82°C. only about 25% of the activity remained.

In histochemical experiments acetone-fixed frozen sections were incubated in the substrate at 6, 37, 61 and 82°C. Activity was distinctly lessened at 6°C. and greatly enhanced at 61°C., as compared with results obtained at 37°C. At 82°C. a great reduction in activity

was found. In the substrate mixtures incubated at the higher temperatures, a white precipitate was noted at the end of the incubation period. This precipitate was a lead salt (probably phosphate), for it was blackened by sulfide.

Inhibition by sodium fluoride. The amount of sodium fluoride sufficient to produce a significant inhibition was studied with frozen sections of liver blocks fixed in 10% formol-saline; incubation times were 45 and 100 min. Concentrations of sodium fluoride down to 0.02 mg. per 10 ml. of substrate (M/17.000) gave a distinct inhibition. On the other hand, sodium fluoride incorporated at a concentration of 2 mg. per 10 ml. in the formol-saline fixative (M/170) did not inhibit enzymatic activity of frozen sections.

Effect of various other chemicals. In frozen sections, ferricyanide (0.01M), ascorbic acid (0.04M) did not affect the enzymatic reaction. Manganous sulfate (0.01M) as recommended by Moog (1943) could not be tried under our conditions because it gave a heavy precipitate of lead sulfate when mixed with the substrate. Ammonium hydroxide (5 drops of a concentrated solution in 20 ml. of water) entirely inactivated the enzyme after an exposure of 20 minutes (acetone-fixed frozen sections). Hydrogen peroxide (0.01M) had no influence on the activity of homogenates, while cysteine (0.01M) increased activity by more than 50%. As the Folin-Ciocalteu reagent is reduced by cysteine, activity was determined with a method for phosphorus. Three ml. of a 0.5% aqueous saponin solution added to 10 ml. of substrate had no influence on the color development of frozen sections.

Extraction of lead phosphate. The cytochemical method was followed to the point just before transferring the sections to ammonium sulfide. They were then kept for 2 hours in distilled water. No loss of color development could be observed.

Discussion

Before attempting to correlate chemical and cytochemical data we wish to emphasize that results cannot be compared directly. One reason for this is that different substrates were employed in the chemical and cytochemical methods. Another reason is that the factors controlling the penetration of the substances used in the cytochemical technic are poorly understood. Penetration through cellular structures and various adsorption phenomena can be influenced in unpredictable ways by temperature and chemical agents.

Effect of fixatives. Our chemical data agree in general with those presented by Emmel (1946) and by Stafford and Atkinson (1948) on the marked decrease in enzymatic activity after fixation in formol-

saline, acetone and 80% alcohol. As it was impossible to prove extraction of acid phosphatase by these fixatives, the decrease in activity seems to be due exclusively to inactivation of the enzyme. In the case of formol fixation, *in vitro* results speak in favor of this interpretation (Emmel, 1946; Abul-Fadl, 1947), but for alcohol fixation they are in contradiction with those of the second author. Emmel's figures were higher than ours, possibly because he worked with homogenates which were dialyzed to remove formol. Gomori (1941) showed the marked reduction of enzymatic activity after acetone fixation by comparing the reactions of fixed and unfixed tissue. The higher enzymatic activity found chemically in tissues fixed for a short period of time (2 hours) seems to indicate that inactivation progresses with time.

The effect of acetone and alcohol may explain the findings of Bartelmez and Bensley on the negative reaction of Schwann cells in fixed material as compared with positive reactions in unfixed tissue.

All other fixatives studied inactivate acid phosphatase to a greater extent both chemically and cytochemically. When interpreting histochemical results obtained with the acid phosphatase technic, one should not forget that glycerophosphate, as compared with phenylphosphate, is poorly attacked by acid phosphatase.

Effect of the histological procedure. Although our experiments were conducted in a different manner, we agree with Stafford and Atkinson on the decrease in enzymatic activity of blocks subjected to conditions similar to those of the histological procedure. This great lowering of activity can be demonstrated also cytochemically.

On the basis of both chemical and cytochemical results we disagree with Gomori's statement that fixation in acetone protects the enzyme from the inactivating effect of alcohol. Our results indicate that alcohol treatment after acetone fixation causes considerable inactivation of the enzyme. This suggests that alcohol should be eliminated from the schedule altogether. The experiments with homogenates show that embedding will inactivate a large portion of the enzyme. We are faced with several inactivating factors that together can account for a great part of the difficulties encountered in the method for acid phosphatase.

Extraction of enzyme and of lead phosphate. We were able to confirm chemically and histochemically the findings of Bartelmez and Bensley on the extraction of acid phosphatase from fixed tissue. Results were conclusive only after 5 hours of extraction. The fact that only 5% of the enzyme could be extracted by water leads us to think that extraction is not a very important factor in the histo-

chemical method. Under the conditions of our work we could not verify the removal of lead phosphate by water from sections. A slight decrease in color intensity was seen after 10 minutes in absolute alcohol plus 110 minutes in xylene or after 120 minutes in absolute alcohol, in partial agreement with the observation of Moog (1943). Xylene, however, may attack lead sulfide, and to prevent this, sections should be cleared in gasoline or tetrachloroethylene and mounted in Clarite dissolved in the same solvents (Gomori, 1945).

Effect of temperature on enzyme reaction. Our chemical results are in agreement with the histochemical ones, pointing to a certain thermostability of the enzyme. The optimal temperature of enzymatic activity seems to lie between 37 and 61°C. It is possible that carrying out the cytochemical method at a temperature closer to the optimum would contribute to better results. We did not study chemically the effect of temperature on enzymatic activity of fixed tissue, but the agreement between the chemical and the visible histochemical results was good. The fact that at 6°C. the histochemical reaction is decreased in intensity and that saponin does not affect the reaction speaks against a process of mere adsorption.

Apart from the difference in material studied, it is possible that this relative thermostability of the enzyme and its resistance to certain agents, together with adsorption of lead, may explain Lassek's findings (1947). When a process of adsorption is suspected, control sections incubated in the presence of fluoride or without glycerophosphate should be used, and only the difference between the control and regular sections should be considered to be due to enzymatic activity.

Effect of sodium fluoride and other chemical agents. In agreement with Wolf *et al.* (1943), we have shown cytochemically a pronounced inhibition of the reaction by as little as $M/17.000$ of sodium fluoride. In a formol solution, used as a fixative, sodium fluoride did not inhibit the cytochemical reaction. This can be explained by the reversibility of fluoride inhibition (Belfanti *et al.*, 1935).

We could not confirm the results of Moog (1943, 1944) in respect to the histochemical inactivation of enzymatic activity by ferricyanide or the enhancement of the reaction by ascorbic acid and manganous sulfate. Lassek (1947) claims to have obtained cytochemical reactions after the treatment of sections with hydrochloric and nitric acids, ammonia, hydrogen peroxide, phosphotungstic acid, mercuric chloride and picric acid. The effect of these substances on acid phosphatase activity should be checked by test tube experiments, using nervous tissue as the source of the enzyme. In our experi-

ments, ammonia treatment of sections completely inactivated the enzyme histochemically, in agreement with the data of Bamann and Diederichs (1934).

Recently, Doyle (1948) studied the effect of ethyl and methyl alcohol and acetone fixation, temperature of fixation, paraffin embedding and freezing-drying on acid phosphatase in the appendiceal lymph nodes of the rabbit. Our results, although in different material, agree with his.

Concluding Remarks

The histochemical technic for acid phosphatase, in its present form, is far from being an optimal one. The use of glycerophosphate as a substrate diminishes its sensitivity. The great inactivation produced by fixation, alcohols and embedding, are factors contributing to poor results. These factors may explain, at least in part, the inconsistency of its results.

In general, our results seem to point to the specificity of the method. Comparison with results of other workers is difficult in view of the difference in tissues studied. The possibility of the extraction of the enzyme, although very slight under our experimental conditions, may deprive the method of its strict localizing value, as asserted by Bartelmez and Bensley.

Further study is desirable on this subject with the aim of devising a modification of the procedure which would assure good fixation and preservation of the enzyme.

In the present state of our knowledge it seems that the main points to be observed for optimal results are fixation in chilled acetone, dehydration in the ice box and exposure to the temperature of the paraffin oven for the shortest possible length of time.

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BIBLIOGRAPHY

Abul-Fadl, M. A. M., and King, E. J. 1947. Inhibition of acid phosphatases by formaldehyde. Biochem. J. **41**, xxxii (Proc.).

Bamann, E., and Diederichs, K. 1934. Trennung der beiden isodynamen phosphoesterasen tierischer organe durch ein selectives inactivierungs-verfahren (III Abhand. zur Kenntnis der Phosphatasen). Ber. Deut. Chem. Gesell., **67**, 2019–21.

Bartelmez, G. W., and Bensley, S. H. 1947. "Acid phosphatase" reactions in peripheral nerves. Science, **106**, 639–41.

BELFANTI, S., CANTARDI, A., and ERCOLI, A. 1935. Researches on phosphatases. II. Inactivation and reactivation of phosphatases of mammalian tissues. Biochem. J., **29**, 842–6.

DOYLE, W. L. 1948. Effects of dehydrating agents on phosphatases in the lymphatic nodules of the rabbit appendix. Proc. Soc. Exp. Biol. & Med., **69**, 43–4.

EMMEL, V. M. 1946. The intracellular distribution of alkaline phosphatase activity following various methods of histologic fixation. Anat. Rec., **95**, 159–75.

GOMORI, G. 1941. Distribution of acid phosphatase in the tissues under normal and under pathologic conditions. Arch. Path., **32**, 189–99.

GOMORI, G. 1945. The microtechnical demonstration of sites of lipase activity. Proc. Soc. Exp. Biol. & Med., **58**, 362–4.

GUTMAN, E. B., and GUTMAN, A. B. 1940. Estimation of acid phosphatase activity of blood serum. J. Biol. Chem., **136**, 201–9.

GREENSTEIN, J. P. 1942. Distribution of acid and alkaline phosphatases in tumors, normal tissues and the tissues of tumor-bearing rats and mice. J. Nat. Cancer Inst., **2**, 511–24.

HEINZEN, B. 1947. Acid phosphatase in transected sciatic nerves. Anat. Rec., **98**, 193–205.

KOCHAKIAN, C. D. 1947. The role of hydrolytic enzymes in some of the metabolism activities of steroid hormones. Recent progress in hormone research, **1**, 177–216.

LASSEK, A. M. 1947. The stability of so-called axonal acid phosphatase as determined by experiments and its "stainability." Stain Techn., **22**, 133–8.

MOOG, F. 1943. The use of manganese in the histochemical demonstration of acid phosphatase. J. Cell. and Comp. Physiol., **22**, 95–7.

MOOG, F. 1944. Localization of alkaline and acid phosphatases in early embryogenesis of the chick. Biol. Bull., **86**, 51–80.

STAFFORD, R. O., and ATKINSON, W. B. 1948. Effect of acetone and alcohol fixation and paraffin embedding on activity of acid and alkaline phosphatases in rat tissues. Science, **107**, 279–81.

WOLF, A., KABAT, E. A., and NEWMAN, W. 1943. Histochemical studies on tissue enzymes. III. A study of acid phosphatases with special reference to the nervous system. Amer. J. Path., **19**, 423–38.

INITIAL AND PERSISTING STAINING POWER OF SOLUTIONS OF IRON-HEMATOXYLIN LAKE

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ABSTRACT.—**A study was made of factors affecting the initial staining power and the stability of iron-hematoxylin lake solutions. The findings were applied to the preparation of a superior hematoxylin staining solution. This is made up as follows: in 50 ml. water dissolve, in order, 1.0 g. ferric ammonium sulfate [$FeNH_4(SO_4)_2 \cdot 12H_2O$], 0.8 ml. sulfuric acid, 50 ml. 95% ethyl alcohol, 0.5 g. hematoxylin. Filter the solution to remove the insoluble, white crust of the ferric ammonium sulfate. The solution stains well ten minutes after it has been made. Peak performance is attained within 5 hours, and is maintained for 4 to 8 weeks. Staining time is 3 to 30 minutes. Excess stain can be rinsed off the slide and section by immersion in water, after which destaining, if necessary, can be accomplished with a solution of 50 ml. water, 50 ml. 95% ethyl alcohol, 0.18 ml. sulfuric acid. The slides may or may not be placed next in a neutralizing solution of 50 ml. water, 50 ml. 95% ethyl alcohol, 0.5 g. sodium bicarbonate. They may then be passed through 50 ml. water, 50 ml. 95% ethyl alcohol on the way to alcoholic counterstaining solutions, or through water leading to aqueous counterstains.**

The nuclear stain produced is black, intense and very sharp and has proved to be consistently excellent on a variety of animal and human tissues following a number of different fixatives.

The abundance of nuclear hematoxylin staining formulae on record does not, unfortunately, deny the proposition that there is yet much room for improvement. Many of the methods do have their points of excellence; but a happy combination of these points is not available. Cole (1943) recently presented a digest of methods employed in preparing hematoxylin solutions and prescriptions for their most profitable use as stains, along with a comprehensive bibliography on the subject.

The present work had as its goal the development of a hematoxylin solution that would be easy to prepare; stain nuclei rapidly, intensely and selectively under a variety of conditions; and be de-

pendably consistent in performance over a period of several weeks. Previous experience indicated that the most promise dwelled in the iron-hematoxylin lake.

In an iron-hematoxylin lake solution with laudable initial staining ability, the retention of this property obviously will depend upon the stability of the solution. The task at hand was thus a dual one involving, first, a study of factors determining the initial staining attributes of an iron-hematoxylin lake solution, and second, a study of factors influencing retention of staining power, which is tantamount to a consideration of factors affecting stability. These studies have, in fact, provided the data for formulating an improved stain. Beyond this, they have shed some light upon the chemistry of hematoxylin staining, which subject, however, is outside the scope of this report.

The procedure employed consisted of determining what the factors are that influence the initial and persisting staining power of an iron-hematoxylin lake solution, and then systematically preparing and testing solutions to find the optimum adjustment of each. The hematoxylin used (National Aniline Division, Allied Chemical and Dye Corporation) was Commission certified. Three different lots were employed. Though the color of the crystals varied from a light chocolate brown to a pale straw yellow, all lots yielded similar results. When a high initial content of hematein was desired in the solution, the dissolved hematoxylin was oxidized with mercuric oxide.

Initial staining ability of an experimental solution was determined by staining tests on a wide variety of human material. Adjacent tissue sections were employed whenever direct comparison of staining abilities was essential. Stability of the staining solution was judged subjectively by the amount of film deposited on the surface of the liquid, by the coating on the glass container, by the precipitate formed, by color changes determined with test strips of bibulous paper, and by staining performance. Occasionally, precipitates were dried and weighed. Determinations of pH were made with a Coleman model G pH meter.

Factors Affecting Initial Staining Power and Stability

The concentrations of ferric ion and of hematoxylin or hematein. In aqueous solutions between pH 1.90 and 0.80, it was demonstrated that increasing the ferric ion concentration or that of the hematoxylin or hematein, or both, decreased the stability of the solution. Decrease in persisting staining power was manifested both by fainter staining of nuclei and by darker staining of background with passage of time. On the other hand, the concentrations had to be sufficient to produce

a stain of adequate intensity. Whether the solution was prepared to contain initially only hematoxylin or a high proportion of hematein made extremely little difference in initial staining performance.

The pH of the solution. Increasing the hydrogen ion concentration, with other conditions maintained constant, increased the stability and the persisting staining power of solutions. Three series prepared with different acids—orthophosphoric, sulfuric and hydrochloric—yielded similar evidence. The sulfuric acid series may be used as illustration. The test solutions were made up with 50 ml. water, 0.25 g. hematoxylin, 0.5 g. ferric ammonium sulfate [$FeNH_4(SO_4)_2 \cdot 12H_2O$] and amounts of sulfuric acid (sp. gr. about 1.84, not less than 94% H_2SO_4) varying from zero to 0.5 ml. In solutions of pH greater than 1.50, heavy precipitates formed almost immediately. The precipitate was heavier and formed more rapidly at the higher pH values. Solutions of lower pH contained 0.10, 0.20, 0.25, 0.30, 0.40 and 0.50 ml. sulfuric acid providing pH values of 1.33, 1.08, 1.01, 0.91, 0.83 and 0.71 respectively. The solution of pH 1.33 showed moderate film and precipitate almost immediately which became heavy in 4 hours. The solution stained well at first but was much less efficient—fainter and less selective—at 1 hour and poor at 4 hours. The solution at pH 0.71 showed no evidence of change at first. At 3 days there was a moderate film which was heavy at 6 days and accompanied by a slight coating. Even at 24 days there was no precipitate. Staining was good for three days, after which it slowly declined, but even at 24 days was not as poor as the performance of the pH 1.33 solution at 4 hours. The intermediate solutions yielded stability and staining data that fell in line between these two at the extremes.

It was apparent that the lowest pH values determined the most selective staining, other conditions being comparable. The most tenable generalized statement covering the mechanics of ordinary staining is that cationic dyes stain anionic tissue elements, and anionic dyes color cationic tissue elements, be the process one of adsorption or of chemical union. For a brief discussion of the theory of staining the reader is referred to Conn (1946). The chemical union view was supported by Craig and Wilson (1937). In connection with the action of iron in staining with hematoxylin these authors suggested that a complex is formed whereby iron binds hematoxylin to protein. Regardless of just how the attachment occurs it was abundantly evident in the present work that as pH was lowered and anionic tissue elements were progressively eliminated, the cationic iron-hematoxylin lake progressively became a more selective nuclear

stain, and, eventually, a weaker one. Whatever the substances the lake stains in the fixed nucleus—nucleoproteins or hydrolytic products—some, at least, were still anionic at pH 0.60, the lowest tested.

The pH of the solution played an important role in the intensity of the nuclear stain in initial trials. At a relatively high pH, where the solution deteriorated almost at once, staining was weak. Relatively low pH values resulted in weak nuclear staining, too: for one thing, anionic nuclear elements might have been attenuated to an extreme, and for another, the concentration of the iron-hematoxylin lake probably was low in this circumstance.

The nature of the anions in the solution. Anions in the solution originate from the acid and from the ferric salt. Since the foregoing considerations indicate the desirability of a low pH, a strong or moderately strong acid and the ferric salt of such an acid are necessary. Nitrate was eliminated on the basis that nitric acid is a capable oxidizing agent even in dilute solutions, and it was thought that the life of the hematoxylin solution might be unnecessarily abbreviated by it. Phosphate proved impractical because, while ferric phosphate was soluble at the pH of the staining solution, it precipitated on the section when the latter was rinsed in distilled water. Oxalate failed: solutions were clear, very pale yellow and did not stain. It is probable that the oxalate, by reduction, freed the solution of the chromophore and of ferric ion. Chloride and sulfate remained as the likeliest possibilities, and of the two, sulfate turned out to be the better. Hydrochloric acid or ferric chloride ($FeCl_3 \cdot 6H_2O$) or both were used to introduce chloride ion into the solution, and sulfuric acid or ferric ammonium sulfate [$FeNH_4(SO_4)_2 \cdot 12H_2O$] or both were employed for sulfate. Sulfuric acid and ferric ammonium sulfate were more convenient to handle in preparing the solutions than were hydrochloric acid and ferric chloride. Sulfuric acid was favored also for its nonvolatility. There was some suggestion that chloride ion added to the instability of the solution, but it was not deemed pertinent to the study at hand to confirm this fully at the moment.

The nature of the solvent. Replacement of water as the solvent by a 1:1 mixture, by volume, of water and 95% ethyl alcohol, markedly increased stability of solutions. Initial staining was about the same in the two solvents. The increase in stability conferred by this solvent did not alter the character of the influence exerted by the other factors affecting stability which have been discussed above. Test solutions were made up to contain 25 ml. water and 25 ml. 95% ethyl alcohol. Sulfate was established as the anion of choice. Quantities of sulfuric acid between zero and one milliliter were em-

ployed in determining effect of hydrogen ion concentration. In the appraisal of the effects of ferric ion and of hematoxylin concentrations, 0.25 g. hematoxylin (making the solution approximately 0.014 molar), 0.40 g. hematoxylin (making the solution approximately 0.022 molar), and double these amounts were used in the several possible combinations with 0.35 g. ferric ammonium sulfate (making the solution approximately 0.015 molar with respect to ferric ion), 0.50 g. of the latter (making the solution approximately 0.021 molar with respect to ferric ion), and double these concentrations.

Preparation and Use of the Stain

The foregoing findings cleared the path to the prime objective of this study, namely, the preparation of a better hematoxylin staining solution employing the iron-hematoxylin lake. The solution should be made with a solvent of 1:1 by volume water, 95% ethyl alcohol; sulfate is the preferable anion. The optimum, practical concentrations of hydrogen ion, ferric ion and hematoxylin were still to be determined.

Information gleaned partly from results herein reported and partly from ancillary experiments suggested that ferric ion and hematoxylin react to form a definite compound, iron-hematoxylin lake, and hydrogen ion. The lake, in solution, is the stain. As would be expected, low hydrogen ion, high ferric ion, and high hematoxylin concentrations favor formation of the lake. If there were no other pertinent factors, it would be necessary merely to set conditions that would result in a satisfactory yield of lake. But complications are rife. Solubility of the lake may vary with conditions in the solution. Side reactions are manifest which result in alteration of the lake or in formation of products which detract from the staining performance of the solution. Furthermore, as has been discussed, careful adjustment of the hydrogen ion concentration is essential in order that the stain be both selective and intense. As a result, some compromises were necessary to get sufficient concentration of lake with sufficient freedom from undesirable side reactions with proper hydrogen ion concentration.

A solution made up as follows is the most favorable for the establishment and retention of effective staining power once an adequate supply of iron-hematoxylin has formed:

50 ml. distilled water
0.8 ml. sulfuric acid (sp.gr. about 1.84, at least 94% H_2SO_4)
1 g. ferric ammonium sulfate [$FeNH_4(SO_4)_2 \cdot 12H_2O$]
50 ml. 95% ethyl alcohol
0.5 g. hematoxylin (Nat. Aniline Div.; Commission Certified)

The order given is the most satisfactory one to observe in adding components. The ferric ammonium sulfate dissolves at room temperature in a few minutes with stirring, so that no time is saved by warming the solution since then it must be cooled and restored to volume. The ferric ammonium sulfate should be completely dissolved (except for the white crust on the crystals, which, if present, remains as a residue) before addition of ethyl alcohol, since it dissolves but very slowly afterwards. The sulfuric acid must precede the hematoxylin in order that the hydrogen ion concentration be optimum before the reactants are mixed. The sulfuric acid is handled easily, safely and accurately by pouring the approximate amount required from a small storage bottle into a 5 or 10 ml. beaker, from which the acid can be drawn into a glass syringe and the required volume delivered. The ethyl alcohol should be added before the hematoxylin. The latter then dissolves in a few seconds with stirring. The solution is filtered. The pH at completion is 1.04 ± 0.03.

It is not known when the reaction yielding iron-hematoxylin in this solution reaches equilibrium; but it is apparent that sufficient of the compound is formed within ten minutes to stain very well. In the interval between 10 minutes and 5 hours after preparation of the solution, its staining performance, though submaximal, is nevertheless very commendable and quite satisfactory in most instances. In no case will it fail to give a usable stain, but with some tissues the nuclear stain may be slightly lighter than that yielded by a solution over 5 hours old. After 5 hours, the staining performance is at a peak, and this peak level is maintained for 4 to 8 weeks. During this interval, a slowly progressive change in the solution is mirrored by an increasing intensity of background staining in the tissue, i.e., diffuse staining of elements other than nuclei. At first, the background is unstained. With passage of time it becomes gray-tinged, which coloration gradually darkens. Finally, and presaging the close of the period of usefulness of the stain, the background becomes a dirty yellow.

Staining time varies from 3 to 30 minutes. Longer periods than this, even days, do not give adverse results, but of course they are unnecessary. Following staining, excess of the dye solution can be rinsed from the section and slide by immersing them in two changes of either distilled water or tap water. The gray color in the background can always be removed completely by destaining in the following solution: 50 ml. distilled water, 50 ml. 95% ethyl alcohol, 0.18 ml. sulfuric acid. The pH of this decolorizing solution is 1.67 ± 0.03.

When the staining solution has exceeded its useful span, the nuclei no longer stain intensely enough, and the gray-yellow background is difficult to decolorize. Following destaining, it is sometimes advantageous to place the section in a neutralizing solution of: 50 ml. distilled water, 50 ml. 95% ethyl alcohol, 0.5 g. sodium bicarbonate. Next, the section may be placed in a solution of 50 ml. distilled water, 50 ml. 95% ethyl alcohol on the way to alcoholic counterstaining solutions, or in distilled water on the way to aqueous counterstains.

The volume of the staining solution should not be allowed to become reduced by evaporation by more than 40% of its original volume. Losses up to 40% may be restored by addition of an 80% ethyl alcohol solution. Losses from the acid destaining and alkaline neutralizing solutions may be compensated similarly.

The nuclear stain produced by the method described is black, intense, and very sharp. It has proved to be consistently excellent on a variety of tissues fixed with 10% formalin; Bouin's; Zenker's; and a modification of the latter containing acetic acid, formalin, ethyl alcohol and isopropyl alcohol. The staining solution has been compared with numerous other hematoxylin solutions as to ease of preparation, convenience of use, length of life, and staining results on similar sections and in combination with various counterstains. It has been found superior to all in the writer's judgment. It may be noted that Weigert's iron hematoxylin gives somewhat comparable results over a much briefer span. On the basis of the present work, the limitations in the value of Weigert's stain can be ascribed precisely to certain faults in the composition of the solution.

REFERENCES

COLE, ELBERT C. 1943. Studies on hematoxylin stains. Stain Techn., **18**, 125–42

CONN, H. J. 1946. Biological Stains, *5th Ed.* Biotech Publications, Geneva, N. Y.

CRAIG, RODERICK, and WILSON, CHARLES. 1937. The use of buffered solutions in staining: theory and practice. Stain Techn., **12**, 99–109.

OBSERVATIONS ON THE STAINING OF CORYNEBACTERIUM DIPHTHERIAE

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Abstract.—**Albert's method of staining diphtheria cultures consists of staining a fixed smear for one minute (some laboratories stain for five minutes) with a solution containing toluidine blue and malachite (or methyl) green, washing with water, and applying Albert's iodine for one minute. This procedure is discussed and criticized, and in addition the mechanism of the stain is elucidated. Also, the procedure which involves staining a fixed smear for one minute with Loeffler's alkaline methylene blue solution is discussed and criticized.**

To overcome the objections to the above staining methods, a different method is proposed. This consists of staining a fixed smear with an acid solution of toluidine blue, washing with water, applying Albert's iodine for one minute, washing with water, and finally applying a safranin solution for 15–20 seconds. The theoretical basis for this method is presented.

Mueller and Miller (1946) pointed out that the decreasing prevalence of diphtheria in many localities has led to the development of a generation of bacteriologists with but little experience in the identification of the causative organism. The writer, prior to taking his present position in a public health laboratory, had but superficial experience with the diphtheria organism, and so was definitely a member of this "diphtheritically lost generation". After a year, however, during which a few sporadic cases of diphtheria have occurred in the community, with the attending deluge of throat cultures, the popular Albert's (1920, 1921) and the classical methylene blue methods of staining smears from Loeffler cultures have come to be viewed with certain dissatisfaction. Consequently, a method was worked out for staining diphtheria cultures which eliminated the objectionable characteristics of the above methods.

The objectionable features of Albert's stain and methylene blue have been stated to some extent by other workers, who usually prefer one or the other method, but they may be stated briefly according to the writer's experience as follows:

Albert's Stain. The staining solution is a mixture of toluidine blue

and malachite (or methyl) green in an aqueous solution containing ethyl alcohol and acetic acid. This solution when first prepared yields a rather considerable precipitate, the formation of which not only decreases the staining capacity of the solution, but also, considering the variation in dye content of different preparations of the stain powder, will result in unavoidable variation in the staining capacity of different solutions.

The stain is essentially a granule stain, and as such, stains only the granules with any reliable uniformity. Much too often the diphtheria cell body is stained too faintly to detect the protoplasmic striations (barring), and in fact at times even the outline of the diphtheria organism is indistinct. In Loeffler cultures, therefore, especially to the beginner, confusion can and does arise as to whether the indistinctly stained, frequently occurring, granulated cocci, appropriately spaced, might not be diphtheria bacilli.

Other bacillary forms which produce granules, if the bacteriologist is not conversant with the finer inner structure of the diphtheria cell, and with the arrangement of the organisms in the microscopic field, can very easily be confused with diphtheria bacilli.

Methylene Blue. This stain can be prepared with uniform staining capacity, and it eliminates some of the objections to Albert's stain. Thus, protoplasmic striations (barring) are shown clearly, and contaminating organisms are stained distinctly enough to prevent confusion with diphtheria organisms. The stain, however, demonstrates only well developed granules, and in the case of an organism which does not show protoplasmic striations, and in which the granules have not yet developed to the stage where they are detectable with methylene blue, the organisms will stain solidly and have none of the inner distinctive features of the diphtheria organisms. It might be added in this connection that the granules of the diphtheria organism are composed of nucleic acid and are slowly formed by metabolic cell processes; the initial tiny granules are easily overlooked with methylene blue, but can be shown by a staining method presented in this paper.

From the above observations, it appeared that a stain to be adequate for use on diphtheria cultures, particularly for bacteriologists with little experience, must demonstrate uniformly: (1) the complete morphological outline of the cell body, (2) the presence or absence of protoplasmic striations, and the presence or absence of granules, no matter how small, and (3) stain contaminating organisms clearly to avoid confusion with the diphtheria organism.

Experimental

Inasmuch as Albert's stain will at times demonstrate both protoplasmic striation and granules, it was decided to investigate the mechanism of this stain, with the object of modifying it to fulfill the requirements listed above. In this study, pure cultures kindly supplied by Dr. Martin Frobisher, Jr., together with a local strain, were used.

Two staining solutions were prepared. One contained toluidine blue in the same proportions with acetic acid, alcohol and water as is found in Albert's stain, and the other contained malachite green, also in the same proportions as in Albert's stain. Fixed smears of the diphtheria organism stained with the toluidine blue solution showed that both protoplasmic striations and granules were stained clearly, with the granules being a considerably darker blue than the rest of the cell. However, with the malachite green solution, the cells were stained uniformly and faintly green and showed neither striation nor granulation. When Albert's iodine was then applied to the stained smears, in the case of the toluidine blue stain, no effect was observed on the striations, but the granules appeared to be somewhat darker. The iodine had no effect on the smears stained with malachite green alone.

Another series of fixed smears was then prepared, stained first with toluidine blue, washed and then stained with malachite green. With this treatment, the malachite green removed the toluidine blue from the protoplasm of the cells, which became light green, with only faint differentiation of the protoplasmic striations. The granules, however, retained the toluidine blue. Application of Albert's iodine then changed the granules from blue to black, with additional decrease in the intensity of the malachite green stain in the protoplasm.

The above simple experiments indicate that in the regular Albert's stain, the malachite green is held by the bacterial protoplasm in preference to toluidine blue, whereas the toluidine blue preferentially stains the granules. The function of Albert's iodine appears to be merely one of increasing the intensity of the granule stain, possibly by formation of a black, insoluble iodine-toluidine-blue oxidation product. This supposition is borne out by the fact that in the test tube, a mixture of Albert's iodine and toluidine blue quickly yields a black precipitate. As indicated above, however, the use of iodine as the final reagent is not desirable since it results in a decrease in the staining intensity of the malachite green. This can probably be explained on the basis of acid production in the iodine solution, which would tend to wash out the stain.

In view of these observations, it was felt that replacement of malachite green with a stain possessing greater affinity for the bacterial protoplasm would result in better and more consistent differentiation of the protoplasmic striations. The ordinary safranin used in the Gram stain was chosen. Slides prepared and stained first with the toluidine blue solution, washed and then stained for 30 seconds with safranin, showed that this dye also replaced the toluidine blue in the protoplasm, but left the latter dye in the granules. Further, the protoplasmic striations were very much more distinctly stained than with Albert's method. It was observed, however, that there were somewhat fewer granules in some cells, and it was apparent that the safranin removed the toluidine blue from some of the smaller granules, leaving them, not blue, but instead a darker red than the rest of the cellular protoplasm. To eliminate this replacement of toluidine blue, advantage was taken of the action of iodine on toluidine blue. By applying Albert's iodine for one minute to the preparation already stained with toluidine blue, washing and then staining with safranin, the toluidine blue was "fixed" in even the smaller granules, which then appeared black and even more distinct in the red cell body. It might be added at this point, that Neisser's stain, consisting of methylene blue solution followed by Bismarck brown or safranin, would probably also suffer from replacement of the methylene blue by any possible overstaining with the other dyes.

The staining method developed from the above experiments consists essentially at this stage of staining with the original Albert (1920) formula, washing, applying Albert's iodine, washing, and staining then with safranin. However, Morton and Francisco (1942) showed that increased acidity in granule stains for the diphtheria bacillus resulted in a better differentiation of the cellular structures. To test the effect of increased acidity on the present method, preliminary experiments were made in which acetic acid concentration of the toluidine blue solution was varied from the original concentration of 1.0 ml. per 100 ml. of staining solution, to 2.5, 5.0 and 10.0 ml. Fixed smears stained with these solutions, followed in the usual manner by iodine and safranin, showed no better differentiation with 2.5 ml. of acetic acid as compared to 1.0. With 5.0 ml. however, there was sharper differentiation of some structures, whereas 10 ml. brought no additional improvement.

The experiment was then repeated, using only 1.0 and 5.0 ml. of the acetic acid. Nine different strains of the diphtheria organism (including *mitis*, *gravis*, *intermedius* and *minimus* types) were used. In a majority of cases the 5.0 ml. concentration of acetic acid resulted in

a much sharper differentiation of the protoplasmic striations, a phenomenon which indicates that acetic acid renders the weakly stainable bands in the diphtheria cell even less stainable by the basic dye safranin. Also the increased acetic acid concentration resulted in some cases in a decrease in the staining intensity of the safranin, thus permitting the black granules to stand out more clearly. The granules themselves, however, appeared to be unaltered by the various concentrations of acetic acid used.

The results of all the above experiments thus led to the following formula and technic, which fulfills to the best advantage the three requirements listed in the introduction.

I. Toluidine blue, certified (52% dye content)	0.15	gram
Glacial acetic acid	5.0	ml.
Ethyl alcohol, 95%	2.0	ml.
Distilled water	100	ml.

The stain is ready for use immediately following complete solution. Filtering is not necessary.

II. Albert's iodine

III. Safranin (As prepared for the Gram stain)

Method. Stain smear with I for 1 minute. Wash with water. Apply II for 1 minute. Wash with water. Stain with III for 15–20 seconds. Wash, dry and examine. Protoplasmic striations when present are always distinctly shown, and usually stain red with a light pink to almost colorless band of material between. Occasionally they may stain a reddish brown. Granules are very distinct and stain black, and in cells with no striation, the protoplasm stains a light pink.

The staining method presented above, though original with the writer, cannot, of course be considered a completely new method. Subsequent review of the literature has yielded the information that all reagents used have been used before in different combinations and modifications by earlier workers (Albert, 1920, 1921; Laybourne, 1924; Weiss, 1929; Kemp, 1931), though in no case was the theory behind these modifications presented. Therefore, so far as is known, the proportions and order of the staining reagents presented in this communication, have not been presented before. It is felt that the present method is preferable to either Albert's method or methylene blue, particularly for bacteriologists with limited experience with the diphtheria bacillus, and also for college and university classes in bacteriology for demonstrating with one method the finer structure of the diphtheria cell.

REFERENCES

ALBERT, H. 1920. A new stain for diphtheria bacilli. J. Amer. Med. Assoc., **74**, 28.

ALBERT, H. 1921. Modification of stain for diphtheria bacilli. J. Amer. Med. Assoc., **76**, 240.

KEMP, H. A. 1931. A simple stain for metachromatic granules. J. Lab. Clin. Med., **16**, 593.

LAYBOURNE, R. L. 1924. A modification of Albert's stain for the diphtheria bacillus. J. Amer. Med. Assoc., **83**, 21.

MORTON, H. E., and FRANCISCO, A. 1942. The staining of metachromatic granules in Corynebacterium diphtheriae. Stain Techn., **17**, 27–9.

MUELLER, J. H., and MILLER, P. A. 1946. A new tellurite plating medium and some comments on the laboratory "diagnosis" of Diphtheria. J. Bact., **51**, 743–50.

WEISS, E. 1929. Method for staining polar bodies. J. Lab. Clin. Med., **15**, 170–1.

SOME MODIFICATIONS OF WARMKE'S PERMANENT SECTION-SMEAR METHOD FOR PLANT CHROMOSOMES[1]

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ABSTRACT.—**A modified schedule for making permanent section-smears is given. This incorporates numerous minor changes such as precooling the 1″–1½″ roots for 3 hours at 0°C., fixing in cold Randolph's fixative, using a partial vacuum for ten minutes, thorough washing after fixation, chloroform infiltration, 15–30 minutes in leucobasic fuchsin followed by washing in water for one to four hours, double staining with crystal violet to obtain intense staining of special value in making photomicrographs, and a different method for smearing soft root tips with small chromosomes. Permanent sections stained only with crystal violet, without hydrolysis, can be smeared; this improves the accuracy of counts on difficult material and is useful when rapid routine methods are employed in the study of large numbers of plants.**

The section-smear method of Warmke (1941 and 1946) was used on fixed anthers and root tips of *Lobelia Cardinalis* and satisfactory preparations were obtained. The root-tip tissues of *Agropyron pectiniforme* were found to be much tougher and harder; experiments were undertaken to try to find a method for softening them. The experiments were later extended to improve the staining, smearing, and a simplification of the schedule.

Various diploid and tetraploid species of *Lobelia*, diploid, triploid, tetraploid, and hexaploid plants of *Agropyron*, as well as miscellaneous species including *Asimina triloba*, *Crepis capillaris*, and *Begonia Evansiana*, have been used as experimental material.

Numerous minor changes and improvements were made and wherever possible, less expensive materials were used, such as Randolph's fixative. The root tips precooled at 0°C. were kept living and large-diameter roots such as those of *Agropyron* were cut long enough to avoid adverse physiological effects; roots should be cut at least 1″ to 1½″ long. A more marked shortening of the chromosomes was obtained by precooling for 3 hours rather than for 1½ hours. Thorough washing after fixation improved the softening

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of the tissues. The chromosomes were more intensely stained when kept in the leucobasic fuchsin for 15 to 30 minutes rather than for 10 minutes. The sulphurous acid wash was omitted. Washing for 1 to 4 hours in water, following the leucobasic fuchsin, softened the tissues.

Three staining procedures were employed: the Feulgen reaction, the crystal violet method, and a combined staining schedule, Feulgen and crystal violet. Chromosomes stained with the Feulgen reaction appeared brilliant light purple (cyclamen purple); with crystal violet, they were blue-violet to reddish-purple; and with the Feulgen and crystal violet, the chromosomes were light bluish-purple to deep reddish- or violet-purple. The combined method increased the intensity of staining and the contrast of the chromosomes; a wide range of contrasts was obtained by adjusting the amount of destaining. The chromosomes of some genera absorbed and held the crystal violet more strongly than others. For the preparation of especially good smears or for making photomicrographs, the double stain, Feulgen and crystal violet, was best; the method is described in Schedule A.

For purposes of photomicrography, it is necessary to have high contrast between the stained chromosomes and the clear background. There must also be good differentiation of chromosome structure. In chromosomes stained with the Feulgen reaction, the details of structure are excellent but the intensity of staining is less than with the combined staining schedule; with the latter, every slide is well stained. Chromosomes are usually densely stained with crystal violet but may lack differentiation of structure. The Feulgen reaction chemically stains the desoxyribose-nucleic acid throughout the whole structure of the chromosome and the crystal violet is absorbed. The Feulgen reaction increases differentiation because the internal structure of the chromosome is much better stained than with crystal violet.

If the Feulgen reaction is desired, steps 8 to 10 are omitted, and the ascending series of alcohols are added in Schedule A. In this laboratory, permanent sections stained with crystal violet are used for making rapid, routine counts on large numbers of plants. It has been found that metaphases in these slides can be smeared, even though there has been no hydrolysis; this has increased the accuracy of making chromosome number determinations on difficult material. The procedure is described in Schedule B.

La Cour (1947) has given a general account of recent developments in smearing. Lee (1937) is a valuable source reference for cytological procedures.

Schedule A

This schedule is recommended when especially good permanent preparations are desired showing the range of chromosome size and morphology, or when it is desired to make photomicrographs. The procedure is:

(1) Place 1″ to 1½″ mitotically-active root tips in vials half-full of water in the 0°C. refrigerator for 3 hours. Avoid direct contact of the vials with the cold metal lining of the refrigerator.

(2) Place vials in dish of ice-cold water. Transfer to room temperature and cut root tips ⅛″ to ¼″ long. Fix in vials of cold Randolph's fixative, using a partial vacuum for the first ten minutes. Keep the vials in the 0°C. refrigerator for two hours and then at room temperature for 22 hours. Wash in water for 2 to 4 hours.

(3) Dehydrate in 50% alcohol for ½ to 1 hour, place in 70% for 1 hour, or store until needed. Complete dehydration, and embed by the chloroform method, but avoid excessive heat. Use rubber-parowax embedding and section at 15μ for small and medium-sized chromosomes.

(4) Deparaffinate in xylene for one minute and dioxane for one minute. Pass the slides quickly through the descending series of alcohols to water. Soak in water for one to four hours.

(5) Normal hydrochloric acid at 60°C. for 45 minutes.

(6) Leucobasic fuchsin for 15 minutes. *Agropyron* root-tip metaphases are stained more deeply after 30 minutes but there is an increase in the hardness of the tissues. A few crystals of potassium metabisulfite may be added to the stain.

(7) Wash in water for one to four hours.

(8) Stain in 1% boiled and filtered aqueous crystal violet for 15 minutes. Rinse quickly in water.

(9) Apply 1% iodine and 1% potassium iodide in 80% alcohol for 30 seconds.

(10) Destain in two changes of 95% alcohol for 10 seconds each.

(11) Destain further in dioxane. Clear in two more changes of dioxane for a total of 5 minutes.

(12) Using a pipette-bottle, rinse sections with very thin dioxane-balsam.

(13) Mount in thin dioxane-balsam. Dry slide at room temperature until dry enough for best smearing, about three to ten days.

Root tips of *Lobelia* are much softer than those of *Agropyron*, and different smearing methods must be used for these two genera. For *Lobelia*, tap lightly with a lightweight scalpel or probe, almost directly over the metaphase plate. It is not desirable to free the

cell from the rest of the section. Smear and spread the chromosomes to obtain optimum separation in a thin, flat plate on the same horizontal plane; see Fig. 1B. If too much pressure is used, chromosome structure will be altered.

For root-tip tissues of *Agropyron*, the selected cell must first be freed from the rest of the section by tapping as described by Warmke (1941). Then smear and tap to separate and spread the chromosomes; finally, use sufficient pressure to fix the chromosomes in the same horizontal plane; see Fig. 1A.

There are numerous advantages with this method, when skill has been acquired through practice: the slides are permanent; chromo-

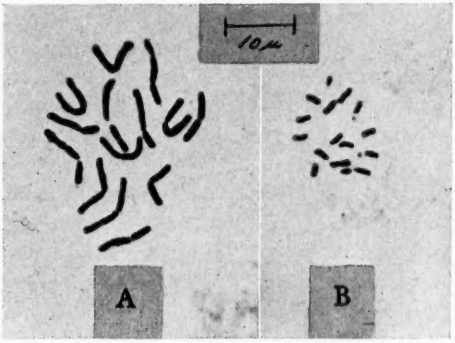

FIG. 1.—Photomicrographs of permanent section-smears of mitotic metaphases in root tips stained with Feulgen and crystal violet, using a combination of Wratten filters No. 60 (green) and No. 22 (deep orange). (A) *Agropyron pectiniforme* R. & S., $2n=14$. (B) *Lobelia Cardinalis* L., inter-varietal hybrid, $2n=16$, diploid complement with two small centric chromosomes. Note the two SAT-chromosomes at 1 and 9 o'clock in A, and at 2 and 4 o'clock in B.

some structure can be observed before and after smearing; staining and fixation are excellent; and large numbers of metaphases are available in each slide [for example in a slide of *Crepis capillaris* ($2n=6$), three satisfactory metaphase smears were quickly obtained in one section].

SCHEDULE B

Many plants are examined cytologically in this laboratory each year and permanent sections stained with crystal violet have proved to be the most satisfactory for rapid routine determinations on root tips. Some of the materials are much more difficult than others. For example, collections of Arctic grasses were examined recently and

a range of chromosome number of $2n=14$ to $2n=140$ was determined. It was found that these permanently-stained crystal violet sections could be smeared fairly well, even without hydrolysis. Such section-smears were particularly valuable, first, in a few slides in which the chromosomes tended to stick or clump together; and, second, in species with a very high chromosome number.

The same procedure is followed as for Schedule A, except that steps (5) to (7) are omitted; and the following steps are added between steps (10) and (11): (i) Destain further in absolute alcohol for 15 seconds and (ii) clear in clove oil for 5 seconds or until sufficiently destained. Step (12) may be omitted, if desired, to shorten the method.

To improve the intensity of staining with crystal violet, leave the slides in each change of 95% alcohol for only 5 seconds each, in absolute alcohol for 5 to 10 seconds, in clove oil for 2 to 5 seconds, and in the first dioxane for 10 seconds. Do not leave for too long a time in the first dioxane since some clove oil is brought over with each slide. Complete destaining and clearing in the 2nd and 3rd dioxane (5 minutes), and mount in dioxane-balsam. Soft root tips can be smeared at once; others should be dried for several days before any smearing is attempted. Best smearing results with routine slides were obtained when the balsam had dried for about one week. The smearing is only partly successful with harder root tips, such as *Agropyron;* good smears can be made with softer materials like *Lobelia.*

The stain is much less intense when crystal violet is used without the Feulgen reaction. The procedure of staining is much more rapid than Schedule A and also more rapid than the Feulgen method. Except in very soft root tips, the metaphases cannot be smeared as well as those done by Schedule A. If rapidity and accuracy in determining large numbers of plants are the only requirements, the crystal violet schedule is more practicable as a routine method. Because of less intense staining and less flattened smears except in the softest root tips, crystal violet smears are not as good for photomicrography as Feulgen-crystal-violet smears.

The following materials could be smeared with the crystal violet method: *Asimina triloba* ($2n=18$); *Crepis capillaris* ($2n=6$); *Lobelia Cardinalis* ($2n=14$, 15, and 16); *Begonia Evansiana* ($2n=26$); *Agropyron ungavense* ($2n=28$); *Calamagrostis* ($2n=140$); *Poa* spp. ($2n=84$, $2n=112$); and *Festuca* sp. ($2n=56$). When the metaphases were smeared, there was a marked improvement in the spreading and separation of the chromosomes. The method was helpful in making

accurate counts on the high polyploid grasses, and in studying chromosome morphology.

The Feulgen, Feulgen-crystal-violet, and crystal-violet staining schedules were used to make permanent section-smears of anthers of *Lobelia Cardinalis*. Staining and smearing results were comparable to those described for root-tip material.

REFERENCES

LaCour, L. F. 1947. Improvements in plant cytological technique. II. Bot. Rev., **13**, 216–40.

Lee, A. B. 1937. The microtomist's vade-mecum, *10 ed., edited by* J. B. Gatenby and T. S. Painter, Blakiston, Philadelphia.

Warmke, H. E. 1941. A section-smear method for plant cytology. Stain Techn., **16**, 9–12.

————. 1946. Precooling combined with chrom-osmo-acetic fixation in studies of somatic chromosomes in plants. Stain Techn., **21**, 87–9.

SUDAN BLACK B—A NEW STAIN FOR CHROMOSOME SMEAR PREPARATIONS[1]

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ABSTRACT.—**Various combinations of Sudan black B (SBB) in some of the lower fatty acids and related acids have been used to stain the chromosomes of smear preparations of onion root-tips prefixed in 2 parts of absolute ethyl alcohol and one part of glacial acetic acid. Temporary preparations were obtained in which the chromosomes and nuclei stained brown but appeared black, when observed with a suitable green filter. Remarkably sharp chromonematic staining was achieved.**

A mixture consisting of equal parts of 1% SBB in 85% lactic acid (LSBB), full strength propionic acid, 1% SBB in 20% formic acid (FSBB) and water added slowly with stirring gave consistently fast and sharp staining with a minimum of dye precipitation. A mixture of equal parts of 1% SBB in 60% propionic acid (PSBB) with LSBB possessed some merit.

A 0.5% solution of SBB in 50% acetic acid yielded an image that resembled the acetocarmine image but did not appear to be quite as useful as the above mixtures. Various solutions of SBB in citric, tartaric, glyceric and glycolic acids were tested. They did not compare favorably with the solutions of SBB in formic, acetic, propionic and lactic acids.

What appeared to be varying chromophoric states of SBB are reported. The blue state did not stain nuclei and chromosomes. They are stained best by the dark red-brown chromophoric state in the presence of adequate water.

INTRODUCTION

The discovery was made recently that Sudan black B (SBB) when dissolved in concentrated lactic acid would stain onion nuclei and chromosomes brown while the nucleoli did not stain (Cohen, 1948). Up to then, SBB had been used chiefly as a fat stain giving a dark blue or a blue-black coloration. Preliminary experiments in which SBB was dissolved in concentrated acetic acid (ASBB) showed that

[1]This work was begun in the summer of 1948 at the Marine Biological Laboratory, Woods Hole, Massachusetts.

this mixture was less useful than the lactic acid mixture of SBB (LSBB). The question then arose whether some other acids related to lactic acid might yield superior staining mixtures. It was believed that such information would help in ascertaining the useful limits of SBB as a chromosome stain. In spite of a tendency towards precipitation from solution, SBB showed decided promise as a sharp nuclear stain when observed with the proper filters. The following series of acids was devised for testing in which due consideration was given to their chemical structure, solubility in water and absence of objectionable odors:

citric $(COOH)CH_2C(OH)(COOH)CH_2COOH$,
d or *l* tartaric $HOOC(CHOH)_2COOH$,
propionic CH_3CH_2COOH, lactic $CH_3CHOHCOOH$,
glyceric $CH_2OHCHOHCOOH$, acetic, CH_3COOH,
glycolic $CH_2OHCOOH$, formic $HCOOH$.

Material and Methods

The usual availability of the common onion bulb, *Allium Cepa*, familiarity with its nuclear appearance (Cohen, 1937) and a large literature for comparison of results favored its use in these experiments. Corroboratory work has been initiated on bean, corn and trillium root-tips.

Sudan black B, product of the National Aniline Division (Certification number NZb-1), was used. The dye solutions were made by boiling and were filtered when cool. Hence, the given dye concentrations in the following solutions are merely approximations. Filtration did not guarantee continued freedom from dye particles since they would reappear in those solutions of acids with high volatility. Except where otherwise noted, 100 mg. of SBB were dissolved in 10 ml. of the organic acid tested to give an approximate 1% solution. In modifying the reported mixtures for use on other material, decrease of the dye content will tend to minimize precipitation of the dye where extremely rapid staining is of secondary importance.

Root-tips and thin slices of scale meristems were fixed in a mixture of 2 volumes of absolute ethyl alcohol and one volume of glacial acetic acid for one-half hour. The material was then transferred to 70% ethyl alcohol. This fixative seemed to preserve the mitotic figures and the details of chromonematic structure better than the mixture incorporating propionic instead of acetic acid.

Maceration of the fixed material in a mixture of equal parts of concentrated HCl and 95% ethyl alcohol was not used, since it seemed to interfere with the subsequent staining. Otherwise the

commonly known manipulations were utilized in making the temporary preparations.

Earlier observations were made using a Wratten X-1 number 11 green filter in combination with a blue daylight filter. Later it was learned that 4 thicknesses of green gift-wrapping cellophane served just as well. The use of a green filter in visual and photographic work is highly desirable since the chromosomes usually appear black on a green background following most of the stain mixtures tried.

Results

Based upon practical utility, the eight organic acids tested as solvents for Sudan black B could be arranged in two groups. Formic, acetic, propionic and lactic acids possessed certain properties that could be adapted for useful chromosome staining in spite of some undesirable features due to the inherent properties of SBB. The remaining acids were not suitable for varying reasons as summarized in Table 1. In all of the acids tested, an adequate concentration of water had to be present in order for suitable staining to occur.

Lactic acid

Lactic acid was the first reagent tested with SBB when chromosomal staining was obtained (Cohen, 1948). Lactic acid 85% was used to make up approximately a 1% solution of SBB (LSBB). When LSBB was used with *N* butyl alcohol in a 9 to 1 ratio as a fixative and stain on fresh root-tips, nuclei and chromosomes stained red-brown, while the nucleoli and cytoplasm did not stain. However, alteration of chromosome structure in which despiralization was most pronounced limited the use of this mixture alone as a fixative. The *N* butyl alcohol was incorporated into the mixture to prevent stain precipitation.

When prefixed root-tips were stained in a mixture of LSBB and *N* butyl alcohol, slightly undersaturated with water in the ratio of 2 to 1, remarkably sharp staining of the chromosomes was obtained as observed through the filter combination. Chromonematic structure and details of coiling during the various stages of mitosis were brought out with diagrammatic clarity.[2]

Preparations incorporating high concentrations of lactic acid are best observed as soon as made. In about a week or so, the stain tends to disappear. (See also Zirkle, 1947). Lactic acid has the advantage of delaying the drying out of temporary preparations. It

[2]Demonstrations were given at the General Scientific Meeting of the Marine Biological Laboratory, Woods Hole, Massachusetts, on August 24, 1948.

has been used in other technics as a dye solvent and mounting medium [Gueguen (1906), Krause (1926), La Cour (1947), Manneval (1936)]. Lactic acid, when mixed with formic or propionic acid or both, seemed to lower their rates of vaporization, thereby permitting utilization of the favorable properties of these acids in SBB formulae.

PROPIONIC ACID

PSBB. When SBB was dissolved in 60% propionic acid (PSBB), a brown mixture was obtained that stained chromosomes a purple-brown. Details of chromonematic structure could be seen. The rapid evaporation of the solvent resulted in excessive precipitation of the dye, especially during the manipulations of smearing. Therefore, the following mixture was tried.

PSBB mixed in equal parts with LSBB. This mixture is a useful one. Mitotic figures in fresh scale meristems were well preserved and stained. Lactic acid alone in high concentrations did not preserve mitotic figures in previously unfixed root-tips. Mitotic figures from prefixed root-tips are equally well stained. Excessive precipitation of the dye seemed to be eliminated in this mixture but the preparations lasted for only a few days due to fading.

Propionic acid alone did not possess any outstanding characteristics. In combination with lactic acid it permitted the preservation of mitotic figures. In combination with formic acid it inhibited cytoplasmic staining as reported below.

ACETIC ACID

ASBB diluted with equal volume of water. When a 1% solution of SBB in glacial acetic acid (ASBB) was diluted with water and the precipitate separated, the red-brown filtrate stained nuclei and cytoplasm brown. The cytoplasm was less heavily stained. The prophase stages in prefixed root-tips were not as clearly differentiated as in the formic and propionic acid mixtures since the nuclear lymph also stained. The nuclei and chromosomes are swollen as one would expect in 50% acetic acid. While the uptake of the dye was moderately fast, warming the preparation as practised in the aceto-carmine technic, hastened the process. However, precipitation of the dye began at the edges of the coverslip as the acetic acid evaporated and the water concentration increased.

SBB is insoluble in hot formalin. When an equal volume of glacial acetic acid was added, the dye was dissolved. The color of the resulting solution was a deep blue. When used on prefixed root-tips, both nuclei and cytoplasm stained brown faintly, if at all, during

the time interval in which successful staining occurred with other mixtures. However, it is of pertinent interest to note that, in the presence of the aldehyde, the blue chromophoric state of the dye was obtained which did not stain nuclei or chromosomes.

FORMIC ACID

FSBB. SBB readily dissolved in 20% formic acid (FSBB) to yield a dark brown solution. In this solution the nuclei stained rapidly, changing from dark brown to black. The cytoplasm also stained but not as deeply. In the case of formic acid, the cytoplasmic staining may be encouraged by the slight amounts of HCHO present. The mordanting effect of formaldehyde upon cytoplasm when used with certain dyes in acid solution is well known (Zirkle, 1947). Formic acid could be considered as coexisting as an aldehyde because hydrogen instead of an alkyl group is linked to the carboxyl group.

FSBB mixed in equal parts with PSBB. From the results obtained with SBB in propionic acid alone, it was considered feasible to try a combination of formic and propionic acids in order to mimimize cytoplasmic staining and still take advantage of the property of formic acid of dissolving SBB in the presence of a large concentration of water. Cytoplasmic staining was greatly inhibited by this mixture. Observations could be readily made upon relational coiling and chromonematic structure of the chromosomes at metaphase. However, this mixture had one of the technical objections encountered in 20% FSBB alone. The rapid evaporation of the solvents resulted in the precipitation of excess SBB. Whereas sealing would obviate some of the difficulty, there was not enough latitude for the necessary manipulation in preparing the smears.

FSBB, PSBB and 42.5% lactic acid mixed in equal parts. Earlier work had shown that lactic acid mounts withstood drying out under ordinary laboratory conditions. The volatility of formic and propionic acids seemed to be decreased in a mixture with lactic acid. While this mixture was found to be useful even though the cytoplasm stained slightly, improvement in the speed of staining and intensity was believed to be possible.

FSBB, LSBB, propionic acid and water mixed in equal parts. Several experiments were made in which the relative proportions of the constituents of the immediately preceding combination were altered. It was found that 0.5% SBB in a formic-propionic-lactic mixture containing approximately 50% water stained nuclei and chromosomes fast and with satisfactory intensity. Also at such

concentration the precipitation of the dye was minimized. The following mixture has given useful results on prefixed onion root-tips, as well as those of corn, bean and trillium: LSBB, propionic acid full-strength followed by FSBB and water added slowly with stirring, all in equal parts.

When this mixture was used as a fixative and stain on untreated root-tips and scale meristems, the internal structure of the chromosomes was preserved and stained with the diagrammatic clarity of a caricature. Interkinetic and early prophase nuclei were preserved and stained with simple, accentuated chromonematic structures requiring cautious interpretations.

TABLE 1. ACTION OF ACIDS UNSUITABLE FOR SBB FORMULAE

Acid	Solubility of SBB*	Color of Solution	Comment
Citric.....	+	Red-Brown	Brown stained chromosomes, badly swollen. Dye ppts., upon successive dilution. Staining of chromosomes slow in diluted solutions. Cytoplasm stains.
Tartaric (d or l)	+	Brown	Destruction of cellular content with gross alteration of nuclear structure. Ppt. of dye upon dilution. Chromosomes stained brown.
50% glyceric	—	———	Position and hydrophyllic activity of hydroxyl groups seem to antagonize favorable action of carboxyl group upon solubility of SBB.
70% glycolic	+	Red	Chromosomes unstained. Diluted to 50%.
50% glycolic	—	———	Heavy ppt. Filtrate pale pink.

*Approximately 100 mg. of SBB dissolved in citric acid 16 g./10 ml. H_2O; in tartaric acid 13.2 g./10 ml. H_2O; in 50% glyceric acid 10 g.; in 70% glycolic acid 10 ml.

DISCUSSION

Sudan black B is a complex polyazo dye with the empirical formula $C_{29}H_{22}N_6$. It is a red brown powder and has been used as a general myelin, bacterial fat and leucocyte granule stain. See indices of Stain Technology, Leach (1938), Hartman (1940), Sheehan and Storey (1947). The solvents previously used for this dye were chiefly 70% ethyl alcohol, 50% diacetin (glycerol diacetate), 50% dioxane and ethylene glycol. Such solutions are deep blue.

Although the information derived from observations on various acid solutions of SBB is suggestive, it does not appear to be sufficiently extensive for the purpose of evaluating the relative importance and the effects produced by the length of the carbon chain and the number of hydroxyl and carboxyl groups upon useful chromosome staining. However, one relationship does seem to emerge, namely,

that the blue chromophoric state of SBB is incapable of staining nuclei and chromosomes.

Apparently the brown chromophoric state of SBB in the presence of an adequate water concentration which stains nuclei and chromosomes can be obtained only by certain organic solvent acids or mixtures thereof. Such solvent acids may produce a solution with a structure of the type most cautiously advanced in a footnote by Zirkle (1940).

The staining of nuclei and chromosomes usually involves salt formation in the presence of adequate amounts of water. SBB is inherently a hydrophobic dye. The rapid evaporation of certain solvent acids from some of the experimental preparations led to an excess of water and the resulting precipitation of SBB. The tendency of SBB to precipitate in the presence of excessive water, the required presence of adequate water to reduce high acid concentrations and to permit chromosome staining presented somewhat of a technical problem, especially, if the speed and intensity of staining were not to be sacrificed by lowered dye concentrations. The lactic-propionic-formic-acid mixtures serve as a temporary expedient for onion material.

The fixation images produced by the various solvent acids in high concentration upon previously unfixed material did not seem to differ except for the one produced by 85% lactic acid in which despiralization seemed to occur in all mitotic stages. Also, the spindle was not preserved. Despiralization seemed to occur as the result of the dissolving away of some nuclear and chromosomal constituents. An extreme condition was encountered in the rounding up of nuclear chromatin in numerous small spherules. This stage was also seen on badly deteriorated preparations which had been prefixed. Moderate despiralization and coincident swelling might be responsible for the accentuated chromonematic images observed.

While certain technics may preserve nuclear and cytoplasmic structures so that greatly esteemed fixation images are achieved, opportunities should not be overlooked to follow previously invisible, intermediate alterations of protoplasmic structures by phase microscopy during fixation under properly controlled conditions as advocated by Duryee (1937).[3]

Such precautions would tend to enhance the assessed validity of certain fixation images.

The novel use of SBB as a stain for chromosome smear prepara-

[3]Also unpublished material which Dr. W. R. Duryee kindly demonstrated and discussed with the writer last summer.

tions may suggest to other workers some additional uses. Bacterial and general plant histological staining have been indicated by certain preliminary experiments too fragmentary to report at this time.

BIBLIOGRAPHY

COHEN, I. 1937. Structure of the interkinetic nucleus in the scale epidermis of *Allium Cepa*. Protoplasma, **27**, 484–95.

COHEN, I. 1948. Fixation and staining of plant nuclei in lacto-sudan black B. Abstract. Biol. Bull., **95**, 253.

DURYEE, W. R. 1937. Isolation of nuclei and non-mitotic chromosome pairs from frog eggs. Arch. exp. Zellforsch., **19**, 171–6.

GUÉGEN, M. F. 1906. Sur le Sudan et l'iode lactique et sur leur emploi dans les colorations combinés. Compt. Rend. Soc. Biol., **60**, 851–3.

HARTMAN, T. L. 1941. The use of Sudan black B as a bacterial fat stain. Stain Techn., **15**, 23.

KRAUSE, R. 1926. Enzyklopädie der Mikroscopischen Technik. Urban & Schwartzenberg, Berlin. (See p. 1549.)

LA COUR, L. 1947. Improvements in plant cytological technique. Bot. Rev., **13**, 216–40.

LEACH, E. H. 1938. Fat staining with Sudan black B. J. Path. and Bact., **47**, 635.

MANNEVAL, W. E. 1936. Lactophenol preparations. Stain Techn., **11**, 9.

SHEEHAN, H. L., and STOREY, G. W. 1947. Improved method of staining leucocyte granules with Sudan black B. J. Path. and Bact., **58**, 336–7.

ZIRKLE, C. 1940. Combined fixing, staining and mounting media. Stain Techn., **15**, 140–53.

————. 1947. Some synthetic resins in combined fixing, staining and mounting media. Stain Techn., **22**, 87–97.

A RAPID TECHNIC FOR DEMONSTRATING MAST CELLS IN MOUSE SKIN[1]

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ABSTRACT.—**A simple rapid technic is described for demonstrating mast cells in mouse skin. The procedure requires about 60 minutes from time of specimen removal until permanent mounting. The steps comprise: (1) stretch-mounting of skin on a cardboard frame; (2) fixing and dehydrating in absolute ethanol for 15 minutes; (3) xylene washing for 10 minutes; (4) absolute ethanol washing for 10–15 minutes; (5) 3–4 minutes in a 0.1% aqueous solution of methylene blue; (6) dehydrating and differentiating in absolute ethanol; (7) clearing in xylene; (8) trimming and mounting. Cell counts may be made immediately, as well as high dry and oil immersion study of cytological detail of mast cells.**

Larsson and Sylvén (1947) have described a method for estimating the number, distribution, and form of mast cells in mouse skin. The method involves a 48-hour fixation in various solutions of basic lead acetate and formaldehyde, followed by serial sectioning, staining in toluidine blue, and cell counting. But the technic is slow, and implies that a specialized and complicated fixation is essential. That this is not the case for skin mast cells is suggested by our own laboratory experience as well as by an examination of the older literature (as referred to in: Lee, 1928; Michels, 1938; Cowdry, 1943). We were therefore led to develop a mast cell technic which would (1) be rapid, (2) not require sectioning, (3) permit ready study of mast cell distribution simultaneously throughout the epidermis, dermis, and subcutaneous connective tissue, (4) permit cell counts. By combining features of technics dating back as far as Ehrlich, a simple satisfactory procedure fulfilling these desiderata was derived, and is described below.

PROCEDURE

Skin (e.g., from the abdominal area) is freed of hair, either by epilation with sodium sulfide or by shaving. The animal is killed by cervical fracture, and the skin is incised from axilla to groin on both sides, and across. The skin patch is retracted from the abdominal

[1]The work reported in this paper is a portion of that being carried on under a grant from the National Advisory Cancer Council of the U. S. Public Health Service.

muscle layers by rapid tearing, and is stretched and pinned to a cardboard frame previously cut to appropriate dimensions (Plate 1, Fig. 1). A frame is used rather than a board so as to facilitate penetration of reagents from both sides.

The frame and attached tissue is immersed in:

(1) *Absolute ethanol for a 15 minute fixation and dehydration period.*

In order to dissolve away soluble fats so that the later aqueous dye solution can more easily penetrate the tissue, the preparation is removed from the alcohol, drained, and immersed in:

(2) *Xylene for 10 minutes.*

To remove the xylene, the preparation after draining is transferred to:

(3) *Absolute ethanol for 10–15 minutes* (*same alcohol as was used for fixation*).

The preparation is then immersed in:

(4) *A 0.1% solution of methylene blue chloride,*[2] *in distilled water for 3–4 minutes.*

In order to wash out the excess methylene blue and to dehydrate the specimen, the preparation is drained and washed in:

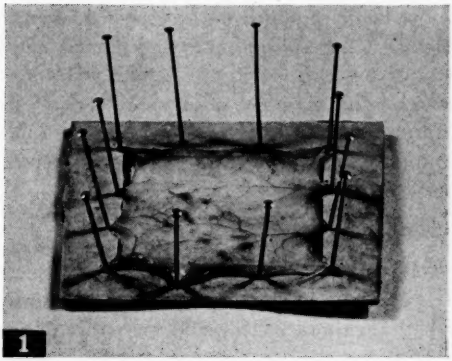
(5) *Two changes of absolute ethanol for a total of 15 minutes.*

This period of time is adjusted depending on the differentiation desired. Ordinarily the mast cell granules in mouse abdominal skin retain a heavy blue-purple coloration, with other cells and tissues transparent or only faintly bluish. If washing is done excessively (i.e., for several hours) the mast cells show a progressive loss of coloration. In practice the appropriate degree of washing may be determined by occasional examinations under a dissecting microscope while the specimen is in its final absolute ethanol bath. If overstaining has occurred, slightly acidified alcohol washing will accelerate de-staining. When the desired degree of differentiation is achieved, the preparation is drained and transferred to:

(6) *Xylene for clearing;* which for mouse abdominal skin takes only a few minutes.

The pins are removed from the now fairly rigid specimen, and the cardboard frame discarded. The specimen is cut down to include the area of interest, and mounted. In most of our preparations we have employed standard microscope slides and 24×50 mm. coverslips, having trimmed the tissue to about 20×45 mm. (Plate 1, Fig. 2). The tissue is mounted flat, with the outer skin surface down. To prevent air bubbles, it is essential that a generous amount of mounting medium be first placed on the slide. The specimen is carefully de-

[2]The lot used by the authors bore Certif. No. NA-27.



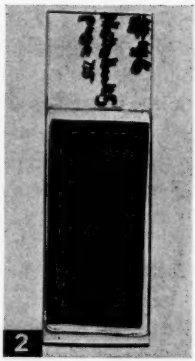


PLATE 1

FIG. 1.—Cardboard frame on which freshly excised abdominal skin patch is pinned prior to immersion in absolute ethanol fixation bath. Approx. Nat. Size.

FIG. 2.—Permanent mount of mouse skin prepared according to the method described.

FIG. 3.—Skin area from Fig. 2 under 6× objective of dissecting microscope, to show general distribution of mast cells. Note hair tufts. Mag. × 250 (approx.).

posited on this, with more medium added on top the specimen, followed by coverslip affixing. Balsam and polystyrene are suitable mounting media. If the tissue has shown any tendency to curl or wrinkle, a coin is placed on the coverslip after mounting, so as to exert a mild pressure during the setting process, although ordinarily the specimen flattens automatically and is ready for microscope study immediately after mounting.

Comment.

Initially a number of tests were performed to determine whether sodium sulfide as used in epilation in any way affected the skin mast cells. Sulfide-epilated skin areas were compared with adjacent skin areas which had been subjected only to shaving, or to no epilatory treatment. In no instance did sodium sulfide induce observable changes in the mast cell picture.

Further preliminary studies revealed that during skin retraction almost all the subcutaneous connective tissue adheres to the dermal tissues. Fixed preparations of both skin and its underlying muscle layers revealed that a negligible number of mast cells remain associated with such muscle layers. It was concluded that the mast cell picture in retracted skin is substantially the true one.

The question of dye penetration is equally important. Focusing on mast cells at various optical depths, it may be established that methylene blue, as applied in this technic, penetrates the whole thickness of the mouse skin, and thus subjects all mast cells of the preparation to staining. In experimental conditions involving pieces of tissue thicker than mouse abdominal skin, the technic should be appropriately modified. For example, in studying the mast cell distribution in the skin area above and on the sides of an implanted tumor, it was necessary to dissect away at least three-fourths of the tumor mass, so as to ensure complete dye penetration, as well as sufficient transparency.

One of the virtues of the procedure here described is that the specimen is ready for study about an hour after removal from the body. Examination of the whole preparation under the dissecting microscope reveals the gross distribution of mast cells; and high resolution study of individual cells may be made under high dry and oil immersion objectives.

It is not the purpose of this note to discuss functional and chemical aspects of mast cell staining. Suffice it to say that the metachromatic granules are the only intracellular structures found to react with the methylene blue, and that the nuclear and cell outlines are discernible only by means of the stained granules (Plate 2).

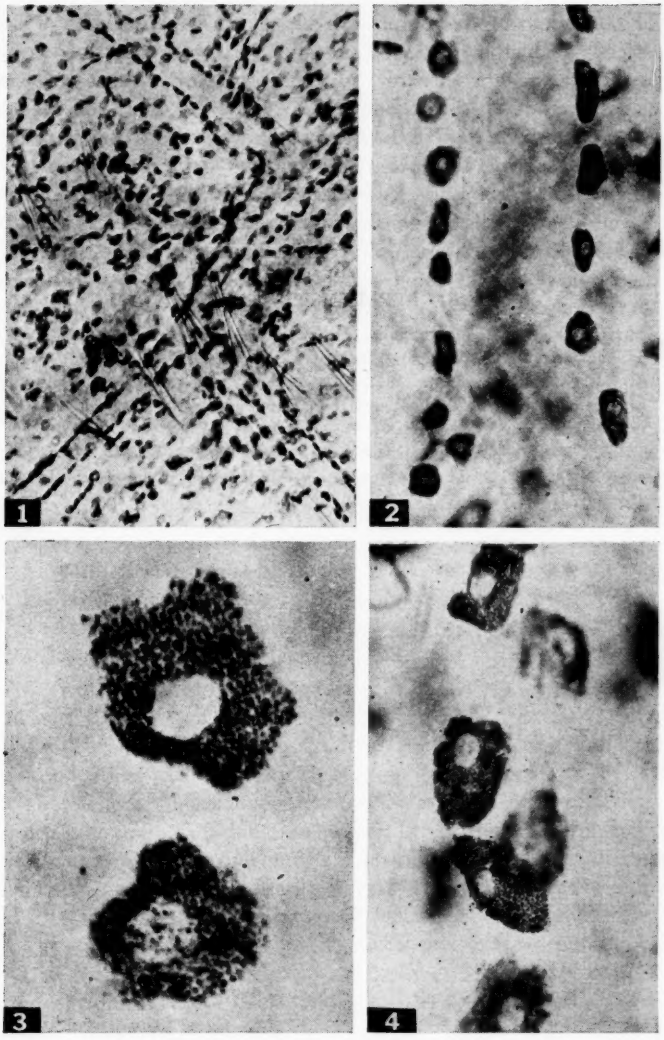


PLATE 2

FIG. 1.—Skin area under 16mm. objective to show location of mast cells in relation to blood channels. Note hair tufts. Mag. × 100 (approx.).

FIG. 2.—Area from Fig. 1 to show enlarged blood channel with peripheral mast cells. Mag. × 400 (approx.).

FIG. 3.—Mast cells from Fig. 1. Oil immersion. Mag. × 2400 (approx.).

FIG. 4.—Mast cells from Fig. 1. High Dry. Mag. × 1200 (approx.).

The technic could be useful both as a quick demonstration of skin mast cells for histology classes, and in the study of effects of various experimental agents on skin and connective tissue mast cells, especially when detailed cell counts are essential, or when the size and shape of cells or the form of the intracellular granules is of importance. With deep focusing a reliable count may be made of apparently all the mast cells of any specified area. The technic also reveals the location of mast cells in relation to such skin structures as vascular channels, hair follicles, areas of experimental injury, etc.

REFERENCES

COWDRY, E. V. 1943. Microscopic Technique in Biology and Medicine. The Williams and Wilkins Company, Baltimore.

LARSSON, L. G., and SYLVÉN, B. 1947. The mast cell reaction of mouse skin to some organic chemicals. I. Estimates of the relative number of mast cells in normal mouse skin. Cancer Research, 7, 676–9.

LEE, A. B. 1928. The Micromotists' Vade-Mecum., *9th Ed.*, London.

MICHELS, N. A. 1938. The mast cells. In Handbook of Hematology, *Edited by* Downey, H. L., Vol. 1, 231–372. Hoeber, New York.

NOTES ON TECHNIC

Ponceau-Fuchsin as a Routine Counterstain

When making Kodachrome photomicrographs of sections stained with hematoxylin and eosin, it was found that the tissues stained by the eosin appeared in the photographs as a shade of orange rather than red. On the other hand it was observed that in the case of sections stained with acid fuchsin, the color of the tissues in the photomicrographs was the same as that in the original sections. Various stains were tried with a view to obtaining a simple counterstain for use with hematoxylin which would not only be more suitable than eosin for color photomicrography, but would also give better differentiation than the diffusely-staining acid fuchsin. The following stains were used: Biebrich scarlet; erythrosin; acid fuchsin; chromotrope 2R; Masson's ponceau-fuchsin mixture. The last stain gave the most satisfactory results and was used as follows:

1. Stain with hematoxylin (Ehrlich, Delafield or hemalum).
2. Leave sections in tap water till blue.
3. Stain with ponceau-fuchsin mixture for 2 minutes (1 part of stock solution diluted with 4 parts of distilled water.)
4. Wash in tap water for 1 minute.
5. Dehydrate, clear in xylene and mount in Clarite.

The ponceau-fuchsin stock solution is made up as follows: acid fuchsin, 0.35 g.; "ponceau de xylidine", 0.7 g.; 1% glacial acetic acid, 100 ml. (Note. Ponceau de xylidine is stated by its manufacturers to be identical with Ponceau 2R; Colour Index No. 79.)

The ponceau-fuchsin counterstain gives good differentiation between muscle fibers (which are stained bright red) and collagen connective tissue fibers (dull pink); it stains more crisply and brightly than eosin and is equally simple to use. It may therefore be employed with advantage as a routine counterstain to hematoxylin.

The stains used were supplied by Messrs. G. T. Gurr, London.

I wish to express my thanks to Professor Francis Davies for his interest and advice.—J. H. Kugler, *Anatomy Department, University of Sheffield, England.*

Delafield's Hematoxylin Combined with Safranin for Anatomical Preparations of Plant Parts

A mixture of ripened Delafield's hematoxylin, made according to the original formula, and 1% safranin O is used for this staining

procedure. The safranin is prepared by mixing 1 gram of dry safranin O with 5 ml. of methyl alcohol. This mixture is then added to 95 ml. of distilled water. A combination in the proportion of 2 ml. of Delafield's hematoxylin and 38 ml. of aqueous safranin was found to be most satisfactory for paraffin sections of stems, leaves, and roots which had been killed in a fluid containing chromic acid. This results in a sharp contrast stain which differentiates cell walls especially well.

A mordanting period of one hour (or more) in 1% chromic acid followed by a thorough washing in tap water may be necessary for material killed in a mixture of formalin, acetic acid and alcohol before proceeding with staining.

Some animal tissues stain reasonably well in this combination. Sections of small intestine for example retain safranin in the mucous cells and the nucleoli. All else retains hematoxylin in varying degrees thus yielding fairly well differentiated preparations. Animal tissues apparently require a somewhat longer staining period (36 to 48 hours) than do plant tissues.

Procedure

1. Take slides down to water and stain in the combination (2 ml. Delafield's hematoxylin and 38 ml. 1% aqueous Safranin O) 12 to 24 hours (overnight is satisfactory).
2. Rinse in tap water.
3. Alcohols—30% and 70%—approximately one minute in each.
4. Differentiate in a saturated solution of picric acid in 70% alcohol —*10 dips.*
5. Rinse in tap water, 30% alcohol, and 70% alcohol.
6. Alcohol, 50% (made up to 0.1% sodium carbonate)—*5 dips.*
7. Alcohol, 95%—2 minutes.
8. Absolute alcohol—2 minutes.
9. Carbol-xylene—2 minutes.
10. Two changes of xylene—5 minutes in each.
11. Balsam.

The stains used were both products of the National Aniline Division, Allied Chemical and Dye Corporation.
Safranin O—Certification No. NS-19
Hematoxylin—Certification No. NH-2

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LABORATORY HINTS
FROM THE LITERATURE

A DEPARTMENT DEVOTED TO ABSTRACTS OF BOOKS AND PAPERS FROM OTHER JOURNALS DEALING WITH STAINS AND MICROSCOPIC TECHNIC IN GENERAL

MICROSCOPE AND OTHER APPARATUS

JELLEY, EDWIN E. **Light microscopy.** *Anal. Chem.*, **21**, 40–4. 1949.

This publication is a summary of papers presented at The Symposium of Light and Electron Microscopy held in Chicago, Illinois, June 10, 11 and 12, 1948. Included in the list are reports concerning the latest improvements in phase, reflecting, ultraviolet, infrared, fluorescence and phosphorescence microscopy. Other new developments mentioned are the variable focus condenser, combined vertical and concentric opaque illumination, hot stages for crystallographic microscopy and the optical dispersion staining method. A list of 150 references is given.—*G. C. Crossmon.*

PHOTOMICROGRAPHY

SHROPSHIRE, R. F. **Oblique photographic illuminator.** *Trans. Amer Micr. Soc.*, **66**, 269–71. 1947.

Two prisms and a concave mirror have been arranged to bring the intense light beam from the lamp used for photomicrography to focus on the surface of an opaque object.—*Virgene Kavanagh.*

MICROTECHNIC IN GENERAL

BURTON, C. J. **Electron microscopy.** *Anal. Chem.*, **21**, 36–40. 1949.

The various technics utilized in preparing specimens for examination with the electron microscope are discussed in this review. (159 references.)—*R. T. Whittenberger.*

COMER, J. J., and HAMM, F. A. **Modified silica replica technique.** *Anal. Chem.*, **21**, 418–9. 1949.

A conical basket, made of 20-mil tungsten wire, is used as the heating unit for vaporizing silica onto microscopic specimens in high vacuum. Three methods are used. In the first silica flakes, which may be obtained by allowing some Ludox (colloidal silica dispersion) to evaporate on a glass slide, are packed in the tungsten basket. In the second method a clean tungsten filament (basket) is dipped four times in Ludox; the filament is allowed to dry before each successive dipping. On a third tungsten filament are hung 4 or 5 quartz fibers about 0.5 mm. in diameter. Three sublimations are carried out in the same vacuum. The relative lengths of time (seconds) required to obtain silica films of comparable thickness are: quartz fibers, 45; Ludox flakes, 15; filament dipped in Ludox, 10 or less. The silica films are alike in all respects.—*R. T. Whittenberger.*

HILLIER, J., KNAYSI, G., and BAKER, R. F. **New preparation techniques for the electron microscopy of bacteria.** *J. Bact.*, **56**, 569–76. 1948.

The organisms are grown directly on the thin supporting membrane of the electron microscope mount in such a way that the organisms are not handled throughout the process except on the membrane. For solid media the following procedure is recommended: flood the surface with sterile distilled water rapidly; place as quickly as possible a drop of 0.5–1.0% collodion solution in amyl acetate on the surface to spread; permit the solvent to evaporate; remove the water with a pipette from under the membrane; check to see that the membrane is thin enough to be visible only at its edges; place a drop of a clean distilled water or saline suspension of bacteria on the membrane with a capillary pipette and withdraw the drop immediately; if the membrane is wet by the suspension, incubate

for 1–3 hours; examine under the light microscope to see when a suitable growth has occurred; select a suitable area and cut out the block of agar below it by cutting straight down with a sharp scalpel; lift the slab of agar with the membrane from the petri dish; slide it under a clean surface of distilled water where the membrane floats on the surface bearing the growth on its upper side; pick up the membrane on the carefully cleaned piece of 200 mesh screen; blot the water from the pores of the screen with hard filter paper; examine under light microscope to select a suitable area for the electron microscope; cut or punch out an area to fit the specimen holder. To apply this method to liquid media, form the membrane on sterile distilled water in a petri dish; evaporate the solvent; pipette off the distilled water; refloat the membrane on the culture media; proceed as above.—*Virgene Kavanagh.*

VAN DUIJN, C., JR. **Substitutes for cedarwood oil.** *The Microscope*, **7**, 91–3. 1948.

The author suggests the use of anisole (methoxybenzene) and methyl benzoate for immersion with oil immersion objectives in place of cedar oil since they are volatile and evaporate from the slide without any residue. Anisole is preferred to methyl benzoate since it is nearer to the required refractive index and is more volatile. Some caution is necessary with uncovered preparations since these reagents tend to dissolve certain dyes such as pyronin and Victoria blue.—*G. C. Crossmon.*

DYES AND THEIR BIOLOGICAL USES

BRANCH, G. E. K., and TOLBERT, B. M. **The spectra of some diaminotriphenylmethane dyes in concentrated sulfuric acid.** *J. Amer. Chem. Soc.*, **71**, 781–7. 1949.

The similarity of the spectrum of malachite green to that of triphenylmethyl ion indicated that, in sulfuric acid, malachite green exists as a triply charged ion with a structure similar to that of triphenylmethyl ion except for two dimethylaminium ion groups at the p and p′ positions. With some phenylated dyes bands were observed in the neighborhood of 700 mμ. These bands were attributed to the addition of a proton to one of the terminal benzene rings, resulting in a carbonium ion compounded of a large number of mesomers, differing from each other in the location of the charge and in the distribution of the double bonds. This mesomeric ion could be considered as a triphenylmethyl ion in which the conjugate system was extended by three more double bonds. Bands attributed to this type of ion were mixed with bands due to the second ion and to ions of the simple triphenylmethyl type.—*R. T. Whittenberger.*

CLAYSON, D. H. F., and PIRIE, D. G. **Observations on the proposed grading of ice cream by a methylene blue test.** *J. Soc. Chem. Ind.*, **67**, 147–50. 1948.

The methylene blue test recommended by the Public Health Laboratory Service for assessing the hygienic quality of ice cream was shown to be unreliable. The weakness of the test is due to the fact that organisms capable of surviving heat treatment considerably more drastic than that required by the Ice Cream Regulations can reduce methylene blue under the conditions of the test. The rate at which they do so depends to some extent on the ingredients used in the ice cream. —*R. T. Whittenberger.*

ANIMAL MICROTECHNIC

BARNES, J. M. **The staining of the duodenal mucosa of rats following the injection of solutions of tannic acid.** *Brit. J. Exptl. Path.*, **29**, 495–500. 1948.

A striking black pigmentation of the duodenal mucosa develops within 7–10 days after a single subcutaneous injection of 1 ml. of 20% tannic acid into the white rat. The staining is due to a deposit of some iron-containing pigment—almost certainly iron tannate—on the adventitial membrane of the capillaries in the duodenal villi. The staining develops within 2–3 weeks to a maximum and then gradually fades over the next 6–10 weeks. Repeated injections of tannic acid intensify the deposition of the pigment. The feeding of iron in excess affects neither the rate of development nor the intensity of the staining. It will also develop in young rats on an "iron-free" diet. By-passing the duodenum by a

gastro-enterostomy will prevent its becoming stained. The phenomenon is discussed briefly, but no satisfactory explanation can be offered.—*J. A. Kennedy.*

BUCHER, GORDON E. **The anatomy of Monodontomerus dentipes Boh., an entomophagous chalcid.** *Canad. J. Res.*, sec. D, **26**, 230–81. 1948.

To homologize the structures of *Monodontomerus dentipes* Boh., a highly modified insect, with those of more generalized insects, the anatomy of adults was studied by the usual methods of serial sections. Dissections were made in a variety of liquids; the most useful were Ringer's solution, 70% alcohol, and a mixture of alcohol and glycerine. A 0.5% solution of acid fuchsin sometimes proved helpful for distinguishing structures. To study the skeleton, the following procedure is recommended: clean in 10% sodium hydroxide; bleach in a mixture of $KClO_3$ crystals, HCl, and alcohol; stain with acid fuchsin. To obtain serial sections, fix in Lebrun's modification of Carnoy's fixative, embed in celloidin, and stain in Ehrlich's hematoxylin and eosin or iron hematoxylin and erythrosin. The anatomy is described and illustrated in considerable detail.—*H. P. Riley.*

LEVADITI, J. C., LEPINE, P., and ANGIER, J. **Etude au moyen de la microscopie en fluorescence, des inclusions intro-cellulaires acidophiles provoquées par les virus de la rage, de la vaccine, de la maladie de Borna.** *Compt. Rend. Acad. Sci.*, **227**, 1061. 1948.

Paraffin sections, after having been passed down to water, are stained for thirty minutes in a solution of two parts thioflavine S to 1000 parts water. They are thus passed rapidly through absolute alcohol and toluene and mounted in a minimum amount of balsam. For photography of filters transmitting, 4000Å gave the best results for fluorescence and 5150Å for visible light.—*T. E. Weier.*

MARSHALL, A. H. E. **A method for the demonstration of reticulo-endothelial cells in paraffin sections.** *J. Path. and Bact.*, **60**, 515–7. 1948.

The following method allows reliable silver impregnation of reticulo-endothelial cells in paraffin sections of formalin-fixed materials: Cut sections 15–20 μ. Without mounting on slides, transfer directly to 2 changes of xylene. On removal of paraffin place sections first in absolute alcohol, then in 50% alcohol. Place sections in the following silver solution for 5–10 sec., prepared by adding, without shaking, 10% $AgNO_3$ to 2 ml. ammonia of sp. gr. 0.880 until there is a slight permanent turbidity. Transfer to 3% formalin in distilled water 2–3 min., moving the section about. Wash in distilled water. Tone in 0.5% gold chloride, wash, fix in 5% sodium thiosulfate, dehydrate, and mount in balsam. Optimum time in the silver solution is usually 7–8 sec. If an especially intense impregnation is desired, after reduction in formalin transfer for 5–10 sec. to a mixture of distilled water 10 ml., silver solution 3 ml. and reduce again. For nuclear staining, wash sections in 2 changes of distilled water after reduction in formalin. Place for 1 min. in strong Ag_2CO_3 solution (details unavailable). Transfer directly to 10% formalin and agitate. Wash, tone in gold chloride, and mount. The cell bodies and processes of reticulo-endothelial cells stain black. In nerve tissue astrocytes and oligodendroglial cells are impregnated. Myeloblasts are not impregnated. Reticulin fibers are usually not impregnated; neither are epithelial elements.—*S. H. Hutner.*

MAYHEW, R. L. **A method for using methylene blue and eosin as a stain for sections.** *Trans. Amer. Micr. Soc.*, **66**, 398–9. 1947.

Mallory's method of staining with methylene blue and eosin frequently understains with the eosin. The following modification has proved to be satisfactory: remove paraffin from sections fixed in Zenker's fluid and hydrate; stain in a solution of 1 g. methylene blue (National Aniline NA-11) and 1 g. borax in 100 ml. distilled water for 3–5 min.; wash off stain in distilled water and pass through 50 and 70% alcohol; differentiate in 85% alcohol to which a few drops of a 10% solution of resin in absolute alcohol has been added quickly; wash in 85% and 95% alcohol to stop destaining; counterstain in a solution of eosin (National Aniline NE-5) 0.5 g. in 100 ml. 95% alcohol for a few seconds. Slides mounted in euparal have retained their color for 16 years. Various brands of dye have been proved satisfactory.—*Virgene Kavanagh.*

RAMSBOTTOM, J. M., and STRANDINE, E. J. **Comparative tenderness and identification of muscles in wholesale beef cuts.** *Food Res.*, **13**, 315–30. 1948.

Fifty of the larger muscles were dissected from the wholesale cuts of three U. S. Good beef carcasses. They were weighed, and their percentage of the total weight in each wholesale cut was recorded. Moisture, fat, and pH determinations were made. Tenderness was determined by committee rating, histological rating, and a shearing device.

Representative samples of both raw and cooked muscles were prepared for histological examination. Small pieces of the raw muscles were fixed in 10% formalin, dehydrated in alcohol, embedded in paraffin, and sectioned. Weigert's elastic connective tissue stain was used to show the distribution of the elastic connective tissue, while Mallory's triple connective tissue stain was used to demonstrate the collagenous connective tissue. From the cooked muscles larger samples of muscles (approximately $1\frac{1}{2} \times \frac{3}{4}$ inches) were sectioned on the freezing microtome and stained with gentian violet and Sudan III.

The beef muscles varied greatly in weight, moisture and fat content, pH, and tenderness. It is apparent from these data that the commercial practice of grouping muscles of similar tenderness should be extended, so that the occurrence of both tough and tender muscles within a roast or steak would be eliminated in so far as it is practical.—*William G. Walter.*

VALADE, PAUL. **Récherches histochimiques sur les lipides de l'écorce cerebrale.** *Compt. Rend. Acad. Sci.*, **227**, 646–8. 1948.

The phosphatides and galactolipids in the brain are demonstrated by a modification of Ciaccio's method which depends upon the insolubility of the major portion of the nerve lipids in acetone and the increase in insolubility obtained through the use of cadmium salts. The method is fairly complicated and the original article should be consulted for details.—*T. E. Weier.*

VALLANCE-OWEN, J. **The histological demonstration of glycogen in necropsy material.** *J. Path. and Bact.*, **60**, 32–7. 1948.

Formaldehyde (4%) is as efficient a fixative for glycogen as Bouin's fluid or absolute alcohol. There is only slight deterioration after 38 days in formaldehyde. This experimental study was carried out on rabbit liver, and the glycogen was stained with Best's carmine.—*S. H. Hutner.*

PLANT MICROTECHNIC

COTTRELL, HELEN J. **Tetrazolium salt as a seed germination indicator.** *Ann. Appl. Biol.*, **35**, 123–31. 1948.

In an attempt to find a chemical test for seed germination that would be more rapid than the standard laboratory test, German workers during 1939–1945 tried 2:3:5-triphenyl tetrazolium chloride and 5-methyl-2:3-diphenyl tetrazolium chloride using maize. As information in England concerning other cereals and legumes was inadequate, an assessment of this method is made using the triphenyl tetrazolium salt and the following method: select seeds at random and steep in tap water for 18 hours; section longitudinally through the embryo; place one half of each in a 7 cm. Petri dish; pour a 1% solution of a tetrazolium salt over the seeds; soak in the dark for 4 hr. at 20° C.; wash with tap water; examine seeds for staining. Viable seeds will be stained. Agreement between this method and the standard germination tests is good in the majority of experiments. The time is reduced by this method but the technic is more difficult and the method is not well adapted to certain seeds.—*H. P. Riley.*

MICROÖRGANISMS

BAKER, H., and BLOOM, W. L. **Further studies on the Gram stain.** *J. Bact.*, **56**, 387–90. 1948.

Escherichia coli, normally Gram-negative, became Gram-positive when a highly viscous solution of desoxyribonucleic acid was added to bacterial suspensions in either distilled water or saline. This Gram-positive reaction was washed away by five washes with distilled water but not by five washes with saline.—*Virgene Kavanagh.*

BAYLISS, M., GLICK, D., and SIEM, R. A. **Demonstration of phosphatases and lipases in bacteria and true fungi by staining methods and the effect of penicillin on phosphatase activity.** *J. Bact.*, **55**, 307–16. 1948.

The Gomori methods for demonstrating acid and alkaline phosphatases and lipases were applied to several bacteria and fungi with considerable success. Alkaline phosphatase activity is demonstrated by precipitation of the phosphate liberated as the calcium salt and its subsequent conversion to cobalt phosphate and then to cobalt sulfide. Acid phosphatase activity is demonstrated by precipitation of phosphate liberated as the lead salt which is then converted to lead sulfide. Lipase activity frees high-weight fatty acids which are precipitated as the calcium salts, then converted to the lead which is demonstrated as the sulfide. —*Virgene Kavanagh.*

COOK, A. H., ELVIDGE, J. A., and HEILBRON, SIR IAN. **Fertilization, including chemotactic phenomena in the Fucaceae.** *Proc. Roy. Soc., Sec. B., London*, **135**, 292–301. 1948.

Detailed instructions are given for methods of studying fertilization in *Fucus serratus, F. vesiculosis, F. spiralis,* and *Ascophyllum nodosum.* These instructions occupy three and one-half pages of text and cannot be condensed.—*T. E. Weier.*

CRUICKSHANK, J. C. **A simple method for testing dye sensitivity of *Brucella* species.** *J. Path. and Bact.*, **60**, 328–9. 1948.

The following is a convenient method for testing the sensitivity to dyes of species of *Brucella*: Place strips of filter paper (Postlip 633) 6 × 0.5 cm. in Petri dishes and sterilize. Pick with sterile forceps and dip one end in a sterile solution of the dye. Dry in the 37° C. incubator overnight. Concentrations of dye solutions used are: thionin, 1:800; basic fuchsin, 1:200; methyl violet, 1:400; pyronin, 1:80; all National Aniline.

For use, lay the strips impregnated with each of the dyes parallel and equally spaced on the surface of a plate of liver agar. Melt a tube of the same medium (12 ml.), cool to 50°C., and pour on top. When the agar is set, dry the plate at 37° C. Add a milky suspension of known strains of *Brucella*, and streak them at a right angle to the filter paper strips, using a spreader 8 mm. in width. The plates, when dry, are incubated at 37°C. in 10% CO_2. Six strains can be accommodated on one 10-cm. plate.

Sensitivity to a dye is indicated by inhibition of growth across a strip.—*S. H. Hutner.*

DOWDING, ELEANOR SILVER. **The spores of Histoplasma.** *Canad. J. Res., sec. E*, **26**, 265–73. 1948.

As there is indirect evidence that *Histoplasma capsulatum* causes benign infection, it was thought advisable to study the spores of Histoplasma in their saprophytic mold phase and in their transition to the parasitic yeast phase. As the usual method of transferring the mycelium by means of an inoculating loop to the mounting medium disarranges the hyphae and scatters the spores of Histoplasma, the following method was used: lower a sterile cover slip over a Petri dish culture until it comes into contact with the aerial mycelium; to examine immediately, place cover slip with spores on a slide containing a drop of cotton blue and lactic acid; for cultural study, place cover slip on a hollow-ground slide containing water with or without nutrient broth or agar and seal with vaseline; leave cultures at room temperature or at 37° C. for about a week. Chlamydospores appear under transmitted light as dark globules because they are immersed in liquid drops, and on lowering the cover slip the drops are pressed out into smears containing conidia. To study the yeastlike phase it is necessary to insure that the spores cannot agglutinate and that they become wet. To bring this about: almost fill depression in hollow-ground slide with Sabouraud's medium; when agar has solidified place drop of plain broth on it; rim depression with vaseline; lower cover slip with spores down over the medium; warm just enough to melt vaseline; place preparation on piece of bent glass tubing on damp filter paper in bottom of Petri dish and keep at 37° C. for a week. The mold stage contains tuberculate spores which have extensions of the spore contents through openings in the wall. The yeastlike phase arises from the hyphae, conidia, or from the tuberculations of the large spores (chlamydospores).—*H. P. Riley.*

KNAYSI, G. **Preliminary observations on germination of the spores of Bacillus mycoides in a nitrogen-free medium and certain properties of the transparent cells.** *J. Bact.*, **55**, 753–7. 1948.

The cytoplasm of the vegetative cells which develop on the nitrogen-free medium does not stain with methylene blue and becomes Gram-negative; the nuclei remain Gram-positive. The cells are highly permeable to neutral red.—*Virgene Kavanagh.*

HISTOCHEMISTRY

PRICE, J. M., MILLER, E. C., and MILLER, J. A. **The intracellular distribution of protein, nucleic acids, riboflavin, and protein-bound aminoazo dye in the livers of rats fed p-dimethylaminoazobenzene.** *J. Biol. Chem.*, **173**, 343–53. 1948.

Rats were fed a synthetic basal diet modified to contain 0.5 or 20.0 mg. riboflavin per kilo with either no azo dye or 0.06% *p*-dimethylaminoazobenzene. Of rats fed the dye in low riboflavin diet 90 to 100% developed liver tumors after 4 months. The livers were fractionated and analyzed for protein, nucleic acids, riboflavin and protein-bound aminoazo dye. Ingestion of *p*-dimethylaminoazobenzene in the diet low in riboflavin increased the protein and desoxypentosenucleic acid contents of the nuclear fraction by 37 and 29%, respectively. Ingestion of the carcinogen in diets containing either low or high levels of riboflavin reduced the protein content of the large granules by 35%, lowered the levels of pentosenucleic acid in the large and small granules by about 40%, and decreased the levels of riboflavin in the large granules and supernatant fluid by 45%. When *p*-dimethylaminoazobenzene was fed, each liver fraction contained protein-bound aminoazo dye. The highest concentrations were found in the small granules and supernatant fluid. Ingestion of a high level of riboflavin lowered the level of the bound dye in each fraction.—*J. E. Kindred.*

SINGER, M., and MORRISON, P. H. **The influence of pH, dye, and salt concentration on the dye binding of unmodified and modified fibrin.** *J. Biol. Chem.*, **175**, 133–45. 1948.

The affinities of films of human fibrin for the acid dye, orange G (certified, purity of sample 89%), and the basic dye methylene blue (certified, purity of sample 84%), have been studied under controlled conditions of pH(2 to 10), ionic strength (0.01 to 0.15) and dye concentration (1 to 124×10^{-6}M). The degree of combination between fibrin and dye was a function of these variables. Treatment of fibrin by heat or formaldehyde resulted in an increase in its affinity for acid and basic dye and a simultaneous or subsequent alteration in its relative affinities for the two dyes. The second effect was correlated with changes in the isoelectric point of the treated fibrin as measured by electrophoretic migration. The maximum binding of anionic and cationic dye at low and high pH, respectively, was much less than the estimated number of charged groups and this binding capacity varied with the ionic strength and concentration of dye.—*J. E. Kindred.*

STAIN TECHNOLOGY

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THE DEMONSTRATION OF A NEW NERVE-CELL ORGANOID

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ABSTRACT.—**A method is described for the demonstration of a new nerve-cell organoid—the binary spheroid systems (Baker bodies). Zenker-formol or acetic-osmium-bichromate materials are postchromed at 37°C. and embedded in paraffin, sectioned and mounted in the ordinary manner. The sections are colored in a 70% alcohol solution of Sudan black B, rinsed in 70% alcohol, counterstained in Mayer's carmalum and mounted in Farrant's medium. After examination the cover may be removed and the Sudan black extracted in 96% alcohol. The sections can then be restained by the azan method of Heidenhain, and other cytoplasmic inclusions can be correlated accurately to the functional states of the spheroid systems.**

A system of binary spheroidal bodies in nerve cells (Baker, 1944) has been studied in both invertebrate neurones and vertebrate sympathetic ganglion cells (Thomas, 1947, 1948), and in Purkinje cells (unpublished). These bodies, originally revealed by Sudan black in frozen sections, have been observed in living neurones after vital staining, and by phase microscopy (Kempson, Thomas and Baker, 1948). They consist of a core of non-lipoidal chromophobe material in a lipoid sheath, exhibit cyclic activity, and influence production of neurosecretory granules (of Scharrer, 1941).

Routine fixatives (such as Zenker-formol) and schedules for paraffin embedding may be employed in the new staining method. The spheroids may be stained with Sudan black, then decolorized with 96% alcohol, and finally restained with a trichrome stain such as the "Azan" stain of Heidenhain. Drawings or photographs of the same cells with both types of staining give, by comparison, the precise relationships between the spheroids and other structures.

The method above shows also the "Golgi zone" in many cells; for

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example, the epithelial cells of small intestine (rat) shown in Fig. 1. The constancy with which results are obtained gives the technic a considerable advantage over the capricious metallic impregnation methods wherein negative results are usually meaningless.

Method

Fixation and embedding.

1. Fix small pieces of tissues in either Zenker-formol, 10%, (Maximow's fluid) or Bensley's A.O.B. solution:

2.5% potassium bichromate (aq.)	8.00 ml.
2% osmium tetroxide	2.00 ml.
Glacial acetic acid	1 drop only

The formalin used in making the Zenker-formol should be neutralized with chalk.

Small pieces should be fixed for 24 hours in either fixative.

2. Rinse quickly in two changes of tap water (5 minutes).

3. Place in a saturated solution of potassium bichromate at 37°C. in an incubator for 3 days.

4. Wash in running water for 1–6 hours.

5. Dehydrate in alcohol, de-alcoholize in ligroin and embed in paraffin wax.

6. Sections are mounted on slides in the ordinary manner using Mayer's egg-albumen-glycerine adhesive.

7. When dry remove the wax in xylene, and take the slides down to 70% alcohol.

8. Shake the slide free from excess 70% alcohol and place it section downwards on a concave staining plate (Gurr, London). Carefully withdraw with a pipette some Sudan black solution from the surface layers of the stock (saturated) solution in 70% alcohol, being careful not to disturb the sediment of undissolved Sudan particles resting on the bottom. Run enough staining solution under the slide to cover the section and allow it to stain for 7 minutes. Any concave staining receptacle, for example, a large clock glass, can be used to provide the means for staining the section in the inverted position.

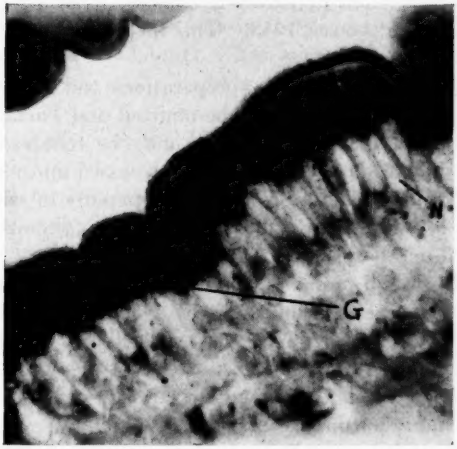
9. Quickly rinse the slide in 70% alcohol for 15 seconds to 1 minute. Agitate the slide vigorously.

10. Place in a jar of tap water and agitate the slide for 1 minute.

11. Stain the nuclei by immersion in Mayer's carmalum solution, 2 minutes.

12. Rinse in tap water.

13. Carefully dry round the section to remove excess water from the slide and mount in a drop of Farrant's medium. Place on a hot plate for 7–10 days to clear and harden thoroughly.



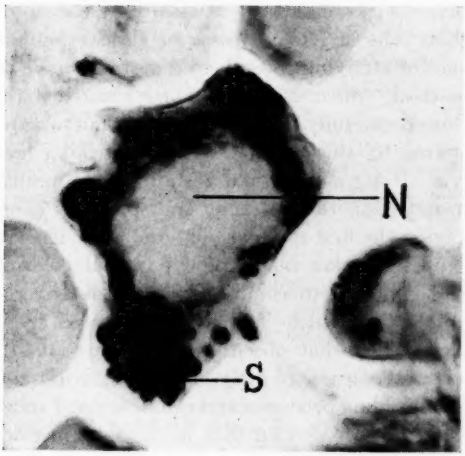


PLATE I

Photomicrographs taken with a Zeiss 2 mm. apochromat, n.a. 1.30, and a 10× compensating ocular.

1. (Top). Paraffin section of the small intestine of a rat. Zenker-formol fixation. Post-chromed. Sudan black technic. N—nucleus. G—Golgi Zone. ×1300 (Approx.)

2. (bottom). Paraffin section of *Helix pomatia* cerebral ganglion. A.O.B. fixation. Sudan black technic. S—spheroid systems. N—nucleus. ×3000 (Approx.)

Alternatively, Sudan preparations may be mounted in balsam as is described by Salthouse, 1943. This method works satisfactorily and gives a harder mount.

If it is desired to destain the preparations and to restain with a trichrome technic, step 11 should be omitted and Farrant's medium used for mounting. The cover glass may be removed readily by soaking the slide in water and the sections passed up to 96% alcohol. All traces of Sudan black will be removed rapidly in strong alcohol. The slide is then downgraded to water and staining commenced as for the trichrome method selected.

The most suitable Sudan black to use is that supplied by the British Drug Houses, London, England (Batch No. 404194). In solution it should be a distinctly carbon black color when a thin film of the solution is viewed with transmitted light (as when the bottle is shaken and the fluid running down the sides is examined). Solutions that are distinctly purplish or bluish black should be rejected as they are never so suitable. Dr. Baker informs me that the present sample of B.D.H. Sudan black as used in the Department of Zoology at Oxford dissolves to less than 0.4% w/v in 70% alcohol.

The ganglion cells of *Helix aspersa* or similar pulmonate proves an ideal tissue for studying the spheroid system of the nerve cell by the above method. Successful results are assured if these instructions are followed carefully. Fig. 2 is a photomicrograph of a *Helix* neurone prepared by this method. It is a striking fact that after decolorizing in strong alcohol and restaining the preparation with hematoxylin and eosin the characteristic spheroids cannot be seen, yet on reapplying the first method they are as obvious as before.

The reason why Sudan black B, a powerful coloring agent for lipoids, should continue to color materials (the spheroid pellicles) in sections of tissues which have been extracted by alcohol and benzene remains somewhat obscure. McManus (1946) has shown that a considerable variety of fatty substances in tissues do survive the ordinary embedding processes and can be stained subsequently by Sudan black. It is also certain that in the short period of staining allowed by the technic Sudan black will only color fatty substances (Lison, 1936).

Postchroming in some degree is nearly always necessary in order to secure good results with the above technic. In this connection Baker (1944) has shown that postchroming retains lipins in tissues and that this process forms an essential step in his acid hematin histochemical reaction (see also Cain, 1947). It seems possible that the phospholipids are held as insoluble chromolipin compounds and that these compounds retain the same affinity as the lipins themselves for dissolving Sudan black. When postchromed tissues are placed in

a 70% alcoholic solution of the coloring agent, the Sudan black deserts its alcoholic vehicle and dissolves in the chrome-lipid compound in which it has a greater solubility. The surrounding cytoplasm is left more or less colorless and a striking differentiation of the spheroids results.

Mr. E. H. Leach, of the Physiology Department, Oxford, has kindly shown me some sections of rat intestinal epithelium prepared by the above method which occasionally show rows of sudanophil droplets passing through the epithelium. These droplets are homogeneously colored and from their general distribution must be considered to be lipoids preserved and retained in the sections during fat absorption. In this connection Mr. A. J. Cain informs me that, according to his unpublished observations, the unsaturated triglycerides of ordinary fat droplets can be rendered insoluble after postchroming, and are then colorable with Sudan black after paraffin embedding. Although penetration of fat-droplets by aqueous fluids is poor, nevertheless the outer shell of large droplets, which is retained and stained black, is far thicker than the sheath of a correspondingly large spheroid and cannot therefore be confused with it. As such shells do not stain with acid hematin it is probable that a chrome compound is not present. The unsaturated bonds have probably been oxidized, with a consequent rise in the melting point of the triglyceride.

I wish to thank Dr. J. R. Baker for his continued help and encouragement during the research which led up to the development of this technic.

REFERENCES

BAKER, J. R. 1944. The structural and chemical composition of the Golgi element. Quart. J. Micr. Sci., **85**, 1.

————. 1946. The histochemical recognition of lipine. Quart. J. Micr. Sci., **87**, 441.

CAIN, A. J. 1947. An examination of Baker's acid haematin test for phospholipines. Quart. J. Micr. Sci., **88**, 467.

KEMPSON, D. A., THOMAS, O. L., and BAKER, J. R. 1948. A simple method of phase contrast microscopy. Quart. J. Micr. Sci., **89**, 351.

LISON, L. 1936. Histochemie Animale. Gauthier-Villars, Paris.

MCMANUS, J. F. A. 1946. The demonstration of certain fatty substances in paraffin sections. J. Path. & Bact., **58**, 93.

SCHARRER, B. 1941. Neurosecretory cells in the central nervous system of cockroaches. J. Comp. Neurol., **74**, 93.

SALTHOUSE, T. N. 1943. Method for the permanent mounting of sections stained for fat. Stain Techn., **18**, 143.

THOMAS, O. L. 1947. The cytology of the neurones of *Helix aspersa*. Quart. J. Micr. Sci., **88**, 445.

————. 1948. A study of the spheroid system of sympathetic neurones with special reference to the problem of neurosecretion. Quart. J. Micr. Sci., **89**, 333.

THIAZIN DYES IN SUPRAVITAL STAINING OF NERVE FIBERS

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ABSTRACT.—**Supravital staining by thiazins of segments of small intestine and mesentery of young dogs was studied with reference to specificity for nervous tissue. Attempts to secure a purer form of methylene blue by alumina adsorption and alcohol elution of the commercial, medicinal dye yielded a product which appeared to be structurally different from the original dye. The treated dye had absorption maxima from 620 to 655 mμ in contrast with 665 for the untreated. Small nerve bundles were stained by the treated dye after 2 to 4 hours of immersion, but staining was always incomplete. Staining by untreated methylene blue was compared with that by the leucobase, thionol, methylene green, toluidine blue, new methylene blue and the azures. It was concluded that the specificity for nerve fibers resides mainly in the $=N(CH_3)_2Cl$ radical, although some specificity appears to be effected by the methyl groups on the trivalent nitrogen, since azure A (dimethyl) and azure C (monomethyl) stained weakly, but thionin did not. Methylene green showed some specificity but stained weakly. The leucobase was less active than the reoxidized dye obtained from it.**

In a previous paper (1947) a technic for immersion staining of nerves with methylene blue was described in which the process could be sufficiently controlled to allow an analysis of factors which affect the reaction. The present paper is concerned with a further study of the staining reactions of interrelated thiazin dyes. The staining procedures used were the same as those described previously except where indicated.

Staining reactions were studied in the following manner. Three types of nerve elements were identified in segments of mesentery: (1) individual nerve fibers in the adventitia of the blood vessels, (2) small collections of non-myelinated fibers in and around the adventitia, and (3) larger bundles covered by a perineural sheath and coursing alongside the blood vessels. The type and intensity of staining of each were recorded as well as the reaction of the mesodermal tissue elements.

[1]Publication No. 523.

In this study 217 segments of mesentery and intestine from 24 puppies or young adult dogs were used.

A. Staining Reactions of Different Samples of Methylene Blue

Nine samples have been used and their effectiveness graded from 0 to ++++. Table 1 lists the results.

Table 1. Staining Reactions of Samples of Methylene Blue

Dye	Date of manufacture	Effectiveness in staining nerve fibers
Grübler	1915	0
Schuchard	1925	++
Cert. No. NA-4 (Nat. Aniline)	1929	+
Cert. No. NA-8 (Nat. Aniline)	1933	++
Cert. No. NA-9 (Nat. Aniline)	1935	++++
Cert. No. NA-11 (Nat. Aniline)	1936	++++
No. 11437	1945	++
No. 11479	1945	+++
Cert. No. CA-23 (Coleman & Bell)	1947	+++

This indicates that not all methylene blue samples are equally effective in staining nerves. At first, this was thought to be due to aging and oxidation of the dye, but now appears more likely due to differences in dye composition. In all recent experiments, dyes of Certification No.NA- 9 or NA-11 have been used.

B. The Purification of Methylene Blue

In the previous paper it was pointed out that there were two phases in immersion staining with methylene blue. First there occurred staining of fat cells, smooth muscle, connective tissue and leucocytes. Later there was selective staining of fine nerve fibers and then bundles. It was suggested that the first or non-specific part of the process might be due to the presence of oxidation products such as the azure compounds. Extraction of azures was attempted to see if the specificity of staining could be improved. Two methods were tried.

(1) *Chloroform extraction.* Holmes (1927–1928) showed that commercial methylene blue contains 10% or more of the azures and described a method for extracting azure B and methylene violet. Solutions of the dye were made slightly alkaline (pH 7.35) with borate buffer and then extracted with chloroform. We modified this procedure by using a phosphate buffer to eliminate the physiologically toxic borate ion and three series of experiments were run: one at pH 5.6, one at 7.3 and another at 8.0. Three or four changes of

chloroform were used in each extraction. Care was taken to use the extracts only after all odor of chloroform had disappeared.

Solutions of purified methylene blue extracted at all three pH levels showed a loss of the specificity for nerve elements and an increased affinity for smooth muscle, connective tissue and endothelial cells, with the most vigorous staining occurring in alkaline solutions. Staining at a pH above 7 tended to produce the "azure type" of reaction in which there was limited staining of nerve fibers and bundles and intense staining of other elements. This suggested that azures were being formed in both controls and extracts. Chloroform extraction reduced the azure content but some azures may have developed, due to the alkalinity and temperature of the experiment (38°C.).

(2) *Chromatographic studies.* Since the results with chloroform extraction were not clear cut, it was decided to try to fractionate the commercial dye by the chromatographic adsorption technic. An adsorbing column was constructed of heavy pyrex tubing having an internal diameter of 3 cm. and a length of 60 cm. It was held in a vertical position by a clamp and ring stand and the lower end plugged with glass wool and a one-hole rubber stopper connected to a suction pump. A funnel was introduced at the top. Two types of adsorbents were tried. The first, activated carbon was found to be difficult to handle in that the dye could not readily be released from the adsorbing surface. Activated alumina (Aluminum Ore Company, Grade F, Mesh 20–40) was then tried and found more satisfactory.

The glass tube was filled with the alumina, a piece of filter paper placed on top of the granules, and the tube closed by the insertion of the funnel at the top. The suction pump was turned on and the column washed with either distilled water or phosphate buffer solutions as suggested by Rieman (1941). Samples of dye (0.25 to 0.30 g.) were dissolved in 100 ml. of either water, ethyl, or methyl alcohol and introduced into the top of the column. In some experiments the dye was sucked downward through the column and in others it was allowed to settle through without suction.

After passage of the dye, the column was washed with one or more liters of either distilled water or phosphate buffer (pH 5.6, 7.0 or 8.0) and then partially dried by sucking air through for 3 to 5 minutes. The alumina was then scraped out of the column in segments and each part placed in a beaker and covered with methyl alcohol, which proved to be an effective eluting agent. In some cases the alcohol was filtered off after 1 hour and in others after 48 hours, and the solution evaporated to dryness on an electric hot plate at 38°C. This

usually took about 24 hours. The dry dye sample was then scraped up and stored in clean vials.

Most samples were subjected to spectrophotometric study (by means of a Beckman spectrophotometer) and then tested for effectiveness in staining nerve elements.

It was found that activated alumina readily adsorbed the dye but without development of a chromatogram (stratification). Repeated washing with from 1 to 12 liters of distilled water or phosphate buffer failed to effect a separation of dye components, hence each column was arbitrarily divided into two or three segments and each segment eluted of its dye content.

Extracted dyes were found to vary in spectrographic absorption maxima from 620 to 655 mμ with little correlation between the level in the column and the maxima. All spectrographic curves had one thing in common; the adsorbed and eluted dye always showed a lower maximum than the control methylene blue (Cert. NA-9, absorption maximum 665 mμ.). In addition the absorption ratio (640/670 mμ.) of all extracts was higher than the control methylene blue. These facts indicated that adsorption on activated alumina changed the structure or composition of the dye.

The assumption of alteration in structure was further borne out in studies of immersion staining. The extracted dyes were observed to stain small nerve bundles after 2 to 4 hours but finer individual fibers did not appear at any time as they did in specimens stained with untreated methylene blue. In five of the extracts, tissue elements other than nerves stained intensely, thereby simulating the azure type of reaction (checked by control studies with azures B, A, and C). The remaining 11 samples were almost completely specific for nerve bundles with little or no staining of other elements.

This latter observation led to an analysis of factors responsible for the change in dye characteristics. Different solvents, eluting agents, and phosphate buffers of varying pH were tested and found relatively unimportant. One fact appeared significant, and that was the duration of contact of the alumina with the dye. Although the solubility of Al_2O_3 is very low (0.0001 per 100 ml.), it seemed that there might be enough alumina dissolved in the solution to form a complex with the dye. Such an effect was tested by adding 0.002 to 0.020% aluminum chloride to methylene blue solutions and examining the staining reactions. No effect was present.

It was concluded that activated alumina probably oxidized or in some other manner altered the molecular structure of the dye. The studies of Holmes and French (1926) indicated that two series of compounds may be derived from methylene blue. One series includes

the azures and thionin and is produced by acid oxidation of methylene blue. The other series includes azures B and A, methylene violet, methyl thionolin, thionolin, and thionol and is the result of alkaline oxidation. Experience with the staining reactions of the azures, thionin, and methylene violet indicated that these were not the same as the 11 samples of extracted dye that stained only nerve bundles. It appeared likely that the unknown derivative represented one or more of the thionols and an attempt was made to obtain these dyes for comparison.

Through the courtesy of Dr. L. Michaelis of the Rockefeller Institute for Medical Research a sample of thionol was obtained. This failed to stain any nerve elements although there was moderate staining of mesodermal elements. This represents a somewhat similar reaction to that of the unknown produced by alumina adsorption and suggests that it is either methyl thionolin, thionolin, or a mixture of the two. When these become available, their staining characteristics should be investigated.

Purification of methylene blue by chloroform extraction of alkaline solutions did not yield a dye which was more specific in staining nerve tissue. Adsorption on activated alumina produced a substance which was almost completely specific for nerve bundles but did not stain individual, isolated nerve fibers well. Neither process gave a product suitable for the usual type of supravital staining.

C. Staining with the Reduced Form of Methylene Blue

An attempt was made to use the reduced form of methylene blue (leucobase) to see if staining could be facilitated thereby. Two methods were used to obtain the leuco-base.

(1) *Reduction with hydrogen and platinum black.* Methylene blue was dissolved in 0.01% concentration in the substrata used previously for immersion staining, and to this was added about 1 g. of freshly prepared platinum black on asbestos fiber. Hydrogen gas was then bubbled through the fluid until it was completely decolorized. Tissue samples were added at this point and allowed to remain for ½ to 1½ hours. After this, they were transferred to plain oxygenated substrate for 2 to 2½ hours more.

With this technic results were poor, and only occasional nerve fibers were stained. Other tissue components showed a moderate degree of staining. The lack of oxygenation of the tissues for 30 or more minutes apparently greatly disturbed the reaction.

(2) *Reduction with sodium hydrosulfite.* The leucobase was prepared also by adding a 2% solution of sodium hydrosulfite ($NaHSO_2$)

drop-by-drop to various concentrations of the dye dissolved in substrate until it was just decolorized. Solutions of 0.01 to 1.6% of dye were used. Precipitated sulfur was filtered out and then the tissue specimens added. Tissue was left in contact with this solution for 5 minutes to 2 hours and then placed in plain oxygenated substrate (without dye) for 2 hours. Staining was observed at half-hour intervals.

This procedure also yielded very poor results in that there was only pale staining of nerve fibers and heavy staining of all mesothelial elements.

In another group of tests the reduced dye was extracted from the aqueous phase with chloroform and then crystallized by evaporation. Long, yellow needles formed, which were rapidly oxidized by air back to the blue dye, and this material was used for staining. No significant difference from controls was noted.

It is concluded that the leucobase is much less effective in immersion staining than the oxidized form of the dye.

D. Staining with Methylene Green, Toluidine Blue and New Methylene Blue

A series of tests was made to determine whether dyes other than pure methyl thiazine compounds would stain nerve fibers.

It was found that methylene green was slightly selective in staining individual nerve fibers and small bundles, which developed a pale green color. It also produced a diffuse green staining of mesodermal elements.

Toluidine blue and new methylene blue did not stain any of the nervous elements but stained the mesodermal structures with varying degrees of intensity.

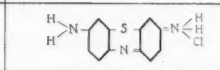
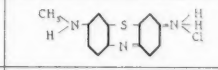
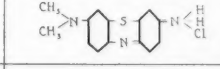
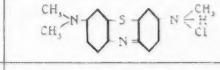
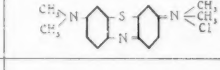
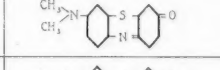
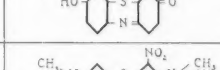
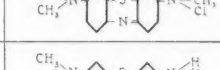
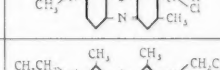
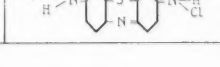
Comment

The methods used to purify methylene blue did not yield any preparation which was as effective for staining nerve fibers as good batches of the commercial dye. Fig. 1 lists the different thiazines studied with their staining reactions and indicates that the nonspecific part of the staining reaction is a property inherent in the thiazine group.

It appears that specificity for nerve fibers resides mainly in the $=N(CH_3)_2Cl$ radical, and when one or both methyl groups are removed (azure B and A), some of the specificity is lost. If the whole radical is removed (methylene violet) there is even further but not complete loss of specificity. Some of the specificity appears to be related to the trivalent N and its two methyl groups. There is still

FIG. 1.

FORMULAE and STAINING PROPERTIES of THIAZIN DYES

	FORMULA	STRUCTURES STAINED		
		Individual nerve fibers	Nerve bundles (without epineurium)	Mesodermal elements
Thionine		0	0	++++
Azure C		0	+	++++
Azure A		0+	++	++++
Azure B		++	+++	++++
Methylene blue		++++	++++	++++
Methylene violet		0	++	+++
Thionol		0	0	++
Methylene green		+	+	+++
Toluidine blue		0	0	++++
New methylene blue		0	0	++
		Intensity of staining graded from 0 to ++++		

some staining of nerve bundles as long as one or two methyl groups are attached to this radical. When all four methyl groups from both radicles are absent, no staining of nerve fibers occurs.

The studies involving staining with leucomethylene blue were unsatisfactory because of the difficulty of maintaining the dye in the reduced form. The presence of air, oxygen, or tissue rapidly converted the dye to the oxidized form and it was impossible to make accurate observations regarding the actual staining process.

Staining by the immersion technic is still unsatisfactory for general neurologic research in that the reaction is not uniform, nerve bundles covered by epineureum stain hardly at all, and penetration of the dye is limited. Perfusion of the living animal as done by Feindel, Allison and Weddell (1948) may prove to be more satisfactory.

We wish to acknowledge our indebtedness to Professor Chester J. Farmer for his aid in the spectrophotometric determinations, and to the Biological Stain Commission for financial aid.

BIBLIOGRAPHY

FEINDEL, WILLIAM H., ALLISON, A. C., and WEDDELL, G. 1948. Intravenous methylene blue for experimental studies on the central nervous system. J. Neurol., Neurosurg., and Psychiatry, **11**, 227–42.

HELLER, RICHARD E., THOMAS, R. W., and DAVENPORT, H. A. 1947. Staining nerve fibers with methylene blue. An evaluation of variables used in an immersion technic. Stain Techn., **22**, 111–18.

HOLMES, WALTER C. 1927. Subsidiary dyes in methylene blue. Stain Techn., **2**, 71–3.

HOLMES, WALTER C. 1928. The spectrophotometric evaluation of mixtures of methylene blue and tri-metyl thionin. Stain Techn., **3**, 45–8.

HOLMES, WALTER C., and FRENCH, R. W. 1926. The oxidation products of methylene blue. Stain Techn., **1**, 17–26.

RIEMAN, W. 1941. The quantitative separation of some dyestuffs. J. Chem. Educ. **18**, 131–3.

A METHOD FOR DEMONSTRATING THE PRESENCE OF ALKALINE PHOSPHATASE AND GLYCOGEN IN THE SAME SECTION

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The distribution and relationships of alkaline phosphatase and glycogen has been the subject of study by a number of workers (e. g. Horowitz, 1942; Wislocki and Dempsey, 1945; Johnson and Bevelander, 1946; Pritchard, 1947), and attempts have been made to relate alkaline phosphatase activity with glycogen metabolism.

In some cases, the distribution of alkaline phosphatase and glycogen is similar, and there is difficulty in deciding whether certain cells contain both. Horowitz (1942) remarked upon this in his studies on fetal heads.

It seems that in general, the technical procedure has been to demonstrate the presence of alkaline phosphatase and glycogen in separate sections which are later compared, although Pritchard (1947) has apparently used a combined staining in his studies of the placenta, but gives no technical details.

While engaged in liver studies following feeding experiments on rabbits, I became interested in elaborating a technic which would show alkaline phosphatase and glycogen in the same section.

The following technic has been found to give good results, and depends upon the fact that absolute alcohol is a suitable fixative for both alkaline phosphatase and glycogen.

(1) The tissue is fixed in absolute alcohol, cleared in xylene, embedded in paraffin at 56°C. and sections made.

(2) The sections are treated by Kabat and Furth's modification (1941) of Gomori's technic for demonstrating alkaline phosphatase as far as the stage of treatment with ammonium sulphide.

(3) After removal of the sections from the ammonium sulphide they are quickly rinsed in water, drained well and treated with freshly prepared Best's carmine for 10 minutes.

(4) The sections are differentiated in Best's differentiating fluid, transferred to equal parts of ether and absolute alcohol—to remove the celloidin film, rinsed in absolute alcohol, cleared in xylene, and mounted in balsam.

RESULT

The presence of alkaline phosphatase is indicated by a black or deep brown deposit of cobalt sulphide, contrasting well with the

bright red appearance of the stained glycogen. Nuclei which do not give any phosphatase reaction are stained red, while those which are phosphatase positive[1] show a brownish-red color.

Discussion

The combination of the two technics has to be made in the order given. Reversal of the procedure, i.e. treatment with Best's carmine prior to the demonstration of alkaline phosphatase, is unsuccessful as the glycogen stain will not be retained.

Ehrlich's hematoxylin is not used as a nuclear stain as is usual in Best's method, for the resulting dark blue stain does not allow a distinction to be made between nuclei that are phosphatase positive and those that are phosphatase negative.

Although Best's carmine is generally diluted immediately prior to use (Best's carmine, 2 vol.; concentrated ammonia, 2 vol.; methyl alcohol, 3 vol.), it will often be found that better results will be obtained without diluting the stain.

This method gives very satisfactory results with sections of rabbits' liver, the jet black bile capillaries contrasting well with the bright red glycogen stain. It is felt that the technic could be employed to advantage on other tissue material when there is doubt as to whether certain cells contain both glycogen and alkaline phosphatase. A certain degree of masking of weak cytoplasmic alkaline phosphatase by the stained glycogen is to be expected, and it will therefore be easier to note whether cells containing alkaline phosphatase are free from glycogen rather than the reverse.

REFERENCES

Horowitz, N. H. 1942. Histochemical study of phosphatase and glycogen in foetal heads. J. Dent. Res., **21**, 519.

Johnson, P. L., and Bevelander, G. 1946. Glycogen and phosphatase in the developing hair. Anat. Rec., **95**, 193.

Kabat, E. A., and Furth, J. 1941. A histochemical study of the distribution of alkaline phosphatase in various normal and neoplastic tissues. Amer. J. Path., **17**, 303.

Martin, B. F., and Jacoby, F. 1948. Diffusion phenomenon complicating the histochemical reaction for alkaline phosphatase. *Paper read at meeting of* Anat. Society of Gt. Britain, Nov. 26th., 1948. I. Anat., (*In Press.*)

Pritchard, J. J. 1947. The distribution of alkaline phosphatase in the pregnant uterus of the rat. J. Anat., **81**, 352.

Wislocki, G. B., and Dempsey, E. W. 1945. Histochemical reactions of the endometrium in pregnancy. Amer. J. Anat., **77**, 365.

[1]A positive nuclear reaction may be a genuine one or may be a pseudo-reaction due to a process of diffusion from neighboring structures of high phosphatase activity (Martin and Jacoby, 1948).

COMBINATION OF CERTAIN FLUORANE DERIVATIVE DYES WITH BACTERIAL CELLS AT DIFFERENT HYDROGEN ION CONCENTRATIONS[1]

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While acid dyes have not been widely used for staining bacteria, several suggestions for their use have been advanced (Conn and Holmes, 1926; Maneval, 1941). In general these procedures call for adding an acid to the stain to intensify the combination of the dye with the bacterial cell. McCalla and Clark (1941) have demonstrated greater adsorption of acid dyes at lower pH levels. Conn and Holmes (1926) list a number of dyes derived from fluorane and compare their relative acidic tendencies, color intensities, and other properties. In a study of the influence of the acidic properties of dyes on their combination with bacterial cells at various hydrogen-ion concentrations, the data herein reported were obtained.

Experimental

Heavy suspensions of bacterial cells were obtained by growing the organisms in a peptone-yeast-extract broth. One-gallon bottles containing two liters of broth were inoculated with growth from Roux bottles and aerated vigorously with sterile air; after 24 hours incubation at 30°C. the cells were harvested by centrifugation. Following three washings with neutral distilled water, the cells were suspended in water in a concentration of from 5–30 billion cells per milliliter. All suspensions were stored at 3°C. and the experiments on dye uptake were carried out within 48 hours after harvesting. To test the uptake of the dyes at different hydrogen-ion concentrations, buffer mixtures of $M/100$ sodium acetate and acetic acid were prepared over the pH range 7.0 to 3.0. Throughout these studies all pH determinations were made with the glass electrode potentiometer. Ten milliliters of buffer were placed in a 20 ml. screw cap test tube and one milliliter of the heavy cell suspension added. It was necessary to check the pH value of the mixture after the addition of the cells because of their ability to combine with the buffer and cause a slight decrease in acidity. Four or five milliliters of a 0.003 to 0.001 molar solution of the dye being studied were then added. After

[1]Contribution No. 247, from Department of Bacteriology, Agricultural Experiment Station, Kansas State College.

mixing, the suspensions were allowed to stand at room temperature for 24 hours to reach equilibrium and then centrifuged. The concentration of the dye remaining in the supernatant fluid was then measured by spectrophotometric means. In all cases, control determinations were made on the dye and buffer in the absence of cells, to correct for any differences due to instability of the dye at the lower pH levels as observed by Holmes (1924). The dyes[2] chosen for this study, listed in order of their relative acidity as recorded by Conn and Holmes (1926), were eosin B, eosin Y, erythrosin B, and rose Bengal.

Results

Fig. 1 shows the per cent of the total dye removed from solution at different hydrogen ion concentrations by cells of *Escherichia coli*. It may be observed that rose Bengal, the dye exhibiting the least acid

Table 1. Percent of Total Dye Combining with Bacillus Subtilis Cells at Different Hydrogen Ion Concentrations

pH	Rose Bengal	Erythrosin B	Eosin Y	Eosin B
Suspension I: 2 billion cells per milliliter in 0.001 *M* dyes				
6.31	22	1	17	1
4.73	47	9	22	5
3.83	87	66	40	47
2.90	99	68	93	65
Suspension II: 5 billion cells per milliliter in 0.003 *M* dyes				
6.50	22	1	1	1
5.65	31	3	5	6
5.12	47	14	16	15
4.32	69	37	23	21
4.05	83	57	58	38
3.45	89	59	74	43

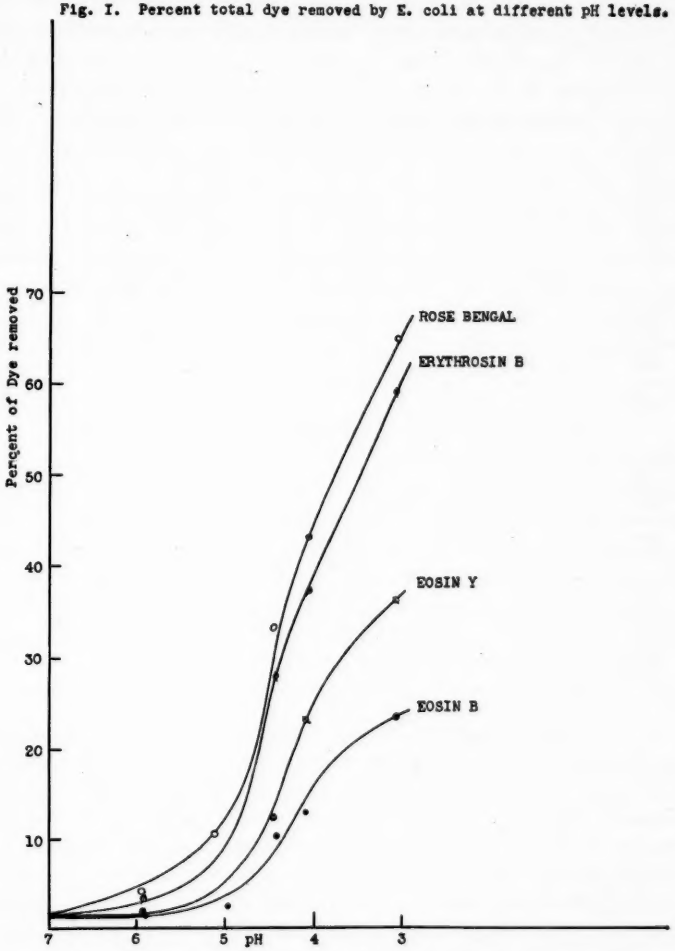
tendency, combined most readily with the cells at all pH levels. Also, the lower the pH level the more readily this dye combined with *E. coli* cells. In general, all dyes studied combined with the bacterial cells in the reverse order of their acidic ionization at all pH levels where measurements were made. Little significance should be attached to the absolute values recorded, since such values will vary with the concentration of cells and the concentration of dye.

In Table 1 are given the percentages of the initial dye combining with *Bacillus subtilis* cells in two experiments in which four different dyes were employed and the ratio between the concentration of cells and dyes varied in the two experiments. The relative position of the different dyes at the different pH levels remained remarkably constant.

[2]All dyes were certified dyes manufactured by National Aniline and Chemical Co.

A summary of data obtained in experiments with four other common bacterial types and the same dyes are recorded in Table 2. Again the order of the removal of dye is related inversely to the acidic character of the dye while combination of dye with cells at different

Fig. I. Percent total dye removed by E. coli at different pH levels.



pH levels is related directly to the hydrogen ion concentration. Again no comparison should be made between the different organisms since the concentrations of cells varied in the different experiments. The relative abilities of different bacteria to take up dyes might be

expected to vary with size, surface, chemical composition, ionization of cellular constituents, etc., none of which was taken into consideration in this study.

TABLE 2. PERCENT OF TOTAL DYE REMOVED BY CELLS OF E. COLI, PROTEUS SP., LACTOBACILLUS SP., AND SARCINA SP. AT DIFFERENT HYDROGEN ION CONCENTRATIONS

pH	Rose Bengal	Erythrosin B	Eosin Y	Eosin B
	(*Escherichia coli:* 20 billion cells per milliliter in 0.003 *M* dyes)			
6.80	1	1	1	1
5.94	3	1	1	1
5.15	10	2	3	1
4.55	33	28	11	10
4.13	43	37	23	12
3.12	65	59	36	23
	(*Sarcina sp:* 15 billion cells per milliliter in 0.003 *M* dyes)			
6.70	1	1	1	1
5.65	1	1	1	1
5.00	21	5	2	3
4.60	40	23	9	17
4.05	49	27	32	18
3.30	94	45	44	21
	(*Lactobacillus sp:* 13 billion cells per milliliter in 0.002 *M* dyes)			
7.00	14	2	1	8
5.42	26	9	1	16
4.49	57	40	3	28
3.62	99	98	75	69
2.92	99	99	90	85
	(*Proteus sp:* 19 billion cells per milliliter in 0.001 *M* dyes)			
7.18	22	11	3	2
5.41	49	28	3	16
4.53	77	69	20	36
3.82	95	97	58	73
2.79	99	99	81	95

It is a well established fact that bacterial cells of most, if not all, species carry a negative charge, which decreases as the hydrogen ion concentration of the suspending medium is increased. This over-all negative charge of the cell would tend to repel particles or ions of like charge, thus tending to prevent their combination with structures of the cell having a positive charge, such as free amino groups or other similar polar groupings in or near the cell surface. These dyes of the fluorane series possess the same general type of molecular structure, yet differ in degree of ionization as acids (Conn and Holmes, 1926). In such a series, the dye with the weakest negative charge (lowest acidic ionization) would be expected to exhibit the least repulsion by the over-all negative charge of a bacterial cell, and a weakly ionized dye should combine more readily with the cell than a more

strongly acid dye. Since the charge on the bacterial cells becomes less as the hydrogen ion concentration of the cell-dye mixture is increased, lowering the pH of such a mixture should facilitate combination of acid dyes with the cell. The results obtained in the present study tend to substantiate the concept that a suspension of bacterial cells in a solution of acid dyes behaves in the theoretical manner suggested.

Additional knowledge of the mechanisms by which dyes combine with the bacterial cell might yield valuable information concerning staining processes, and also help explain the uptake of nutrient materials by the living cell. This study suggests the urgent need for more exact information, particularly on the following points: The influence of various ions carrying an opposite charge to that of the chromophore ion upon the ionization of dyes; the over-all electrical charge of bacterial cells at different pH levels as determined by electrophoresis; and the combining capacities of dyes with bacterial cells at various hydrogen ion concentrations. Further studies along these lines are underway in this laboratory.

Summary

Quantitative determinations of the amount of dye combining with cells of five common bacterial species at different pH levels have been made using four fluorane derivatives, eosin B, eosin Y, erythrosin B, and rose Bengal. Rose Bengal, the least acid of these dyes, combines most readily with the cells at hydrogen ion concentrations from pH 7.0 to 3.0. Adsorption of the dyes in this series was in general inversely related to the degree of acidic ionization of the dye molecule. All dyes combined with the cells more readily at the lower pH levels. A brief discussion of the theoretical implications in staining is given.

REFERENCES

Conn, H. J., and Holmes, W. C. 1926. Fluorescein dyes as bacterial stains: with special reference to their use for soil preparations. Stain Techn., **1**, 87–95.

Conn, H. J., and Holmes, W. C. 1928. Certain factors influencing the staining properties of fluorescein derivatives. Stain Techn., **3**, 94–104.

Holmes, W. C. 1924. Influence of constitutional variation upon the absorption and stability to hydrogen ions of certain halogenated derivatives of fluorescein. J. Amer. Chem. Soc., **46**, 2770–5.

Maneval, W. E. 1941. Staining bacteria and yeasts with acid dyes. Stain Techn., **16**, 13–20.

McCalla, T. M., and Clark, Francis E. 1941. Dye adsorption by bacteria at varying H-ion concentrations. Stain Techn., **16**, 95–100.

A SIMPLIFIED METHOD FOR THE DOUBLE EMBEDDING OF TISSUE

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One of the major advantages of the paraffin method lies in the ease with which either continuous or interrupted serial sections may be prepared. Similarly the absence of distortion represents a very real advantage for the celloidin technic. Brown (1948) has recently remarked that double embedding methods may often be more suitable than either alone, but the technic he has outlined, which is similar to one we have used for some years, possesses the disadvantage that ribboning is impossible.

Because a projected research problem involved the preparation of serial sections from a large number of rat brains, we attempted to modify the double embedding technic to make ribboning possible. We found that reducing the amount of celloidin in the tissue by the complete elimination of one step greatly improved the technic. With the method as outlined below we find it possible to cut blocks (dry) routinely on a rotary type of paraffin microtome at thicknesses varying at least from 5 to 50μ. All of these have ribboned well. Albumen has not proved satisfactory for affixing the sections to the slides but the Masson gelatin technic (Lillie, 1948) with the use of formaldehyde vapors for hardening the gelatin has proved adequate even for sections as large as sagittal sections through an entire rat brain. However, we find the use of fresh gelatin solutions necessary. In order to decrease wrinkling of sections we have found it advantageous to remove the slides from the warming plate as soon as the sections have spread, allow a few minutes for cooling and then drain off the excess gelatin solution. Then the sections are blotted down, flattened with filter paper and placed in the formaldehyde vapors. Among the stains in routine use with us are hematoxylin-eosin, cresyl echt violet, and thionin.

The following timing schedule is suitable for whole rat brains but it can be considerably altered with smaller masses of tissue:

1. Place the formalin fixed brains in 95% alcohol without washing where they remain for one week with two changes of the solution.
2. Transfer to 95% alcohol and ethyl ether (equal parts of each) for one week with two changes.

3. Place in a 3% solution of nitrocellulose in 95% alcohol and ether for one week. [We find it convenient to make up stock solution of 20% as given by Bensley, (1941) and dilute as needed].
4. Allow to remain in a 6% solution of nitrocellulose for one week.
5. Remove block of tissue from nitrocellulose, allow excess to drip off but do not allow surface to become hardened, and place in chloroform overnight.
6. Place in benzene for 48–72 hours with at least three changes.
7. Infiltrate with paraffin for about eight hours. We begin with about 48° paraffin with successive baths in slightly higher melting point paraffin and embed in 53°–55° paraffin. 8 hours.
8. Block and embed as if only in paraffin.

REFERENCES

Brown, J. O. 1948. A simplified and convenient method for the double-embedding of tissues. Stain Techn., **23**, 83–9.

Lillie, R. D. 1948. Histopathologic Technic. The Blakiston Co. Philadelphia.

Bensley, R. R. and Bensley, S. H. 1941. Handbook of Histological and Cytological Technique. U. of Chicago Press. Chicago.

A FIVE-HOUR VARIANT OF GOMORI'S METHENAMINE SILVER METHOD FOR ARGENTAFFIN CELLS

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Abstract.—**Gomori's methenamine silver method for argentaffin cells has been modified and considerably accelerated by almost doubling the silver concentration and raising the incubation temperature to 60°C. Argentaffin cells are selectively impregnated in 3 to 4 hours and the background remains relatively clear up to 4 hours. The contrasts are clearer than with the ammoniacal silver methods of Masson and of Gluckmann.**

In a series of comparisons of the Masson silver method as modified by us[1] with Gomori's[2] recent methenamine silver technic, it was observed in the latter that irregularities in the rate of blackening of argentaffin cells seemed to correlate with temperature variations in the 37°C. incubator employed. As it appeared that it might be possible to shorten the Gomori method so that it could be completed on the same day, it was decided to try incubation in the paraffin oven at about 60°C. and at the same time to almost double the silver concentration.

It was found that very satisfactory demonstration of argentaffin cells in formalin-fixed paraffin sections could be obtained in 3–4 hours at 58°–62°C. When the temperature was raised to 65°C. on one series of sections, impregnation of argentaffin cells was more rapid but silver precipitated very diffusely over the slides. Hence, no further trials were made at this higher temperature.

The material used at this stage was human material removed surgically from stomach, duodenum, ileum, appendix, and colon, and fixed routinely in more or less acid formalin of about 10% dilution. Observations were then extended to stomach and intestine of rabbits, rats, guinea pigs and one dog, fixed in buffered neutral aqueous 10% formalin, as well as in other fluids. The materials fixed in other solutions gave quite variable results and will be included in a later study.

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[1]Laskey, A. and Greco, J. 1948. Comparative study of argentaffin cells of human appendix using modified Schmorl and Masson technics. Arch. Path., **46**, 83.

[2]Gomori, G. 1948. Chemical character of the enterochromaffin cells. Arch. Path., **45**, 48,

For material fixed in neutral formalin, the following procedure can be recommended.

Solutions:

Stock methenamine silver solution: Dissolve 3 g. methenamine (hexamethylene tetramine) in 100 ml. distilled water. Add 5 ml. 5% aqueous silver nitrate. This solution can be stored in a cool dark place for months.

Working solution: To 30 ml. stock methenamine silver solution add 8 ml. Holmes' pH 7.8 borate buffer.[3]

Glassware: The Coplin jars should be chemically clean. Previous silver mirror deposits should be removed with concentrated nitric acid.

Technic:

1. Treat deparaffinized sections with Weigert's iodine solution ($I:KI:H_2O = 1:2:100$)[4], 10 minutes.
2. Bleach with 5% sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$), 2 minutes.
3. Wash in running water, 10 minutes.
4. Rinse in 2 changes of distilled water.
5. Place sections in Coplin jars containing the buffered methenamine silver solution at room temperature and put in 60°C. (paraffin) oven for 3–3½ hours.
6. Rinse in distilled water.
7. Tone in 0.1% gold chloride ($HAuCl_4$), 10 minutes.
8. Rinse in distilled water.
9. Fix in 5% aqueous sodium thiosulfate, 2 minutes.
10. Wash in running water, 5 minutes.
11. Counterstain with 0.1% safranin O in 0.1% acetic acid, 5 minutes.
12. Dehydrate with acetone, clear in xylene, and mount as usual.

Gomori's directions called for 25 ml. of methenamine silver solution, 25 ml. of distilled water and 5 ml. of a borate buffer in which M/5 borax was used. During the earlier part of this work this buffer of Gomori's was used. It was soon found that the M/5 borax crystallized out on standing. It was then decided to substitute Holmes' borate buffers[3]. Usually Holmes' pH 7.8 buffer was used. Although higher pH levels gave more rapid impregnation, results were not as selective, as Gomori already noted, and the silver tended to precipi-

[3]See p. 265 *in* Lillie, R. D. 1948. Histopathologic Technic. The Blakiston Co., Philadelphia.

[4]Gomori (personal communication) has emended the technic, and uses Gram's iodine ($I:KI:H_2O = 1:2:300$).

tate diffusely over the slides. The molar proportion of buffer to silver complex was calculated to be nearly the same as in Gomori's final mixture, but the silver concentration in our final solution was slightly less than twice as high.

In the experimental use of this method incubation times were varied by half hour intervals for 1½ to 4½ hours. At 3 to 3½ hours impregnation was optimal. Argentaffin cells were well blackened and appeared in as great numbers as with longer impregnations. Coarse connective tissue of the submucosa sometimes showed a variable amount of blackening at this time. In 2 to 2½ hours' incubation, partial blackening of argentaffin cells in reduced numbers was seen. At 1½ hours, only cellulose was black. At longer intervals than 3½ hours, besides the connective tissue, the granules of eosinophil leucocytes, nuclei, smooth muscle and surface epithelium became blackened. By 4½ hours a silver mirror had begun to appear on the sides of the slides and Coplin jars.

Gomori's regressive differentiation of over-silvered preparations with weak acidified iron alum solution did not give particularly satisfactory results with our variant. With the shorter incubation periods used in this new technic, it is more practical to repeat the procedure with a briefer silvering time on duplicate sections.

Discussion

This technic in our hands gives sharper differentiation of argentaffin cells with less silvering of background than the Masson technic. The shorter incubation at higher temperatures in our procedure gives us results comparable with Gomori's 12–48 hour, 35–45° technic. Gluckmann[5] used higher temperatures in the Masson technic and reduced the incubation period to 5 to 10 minutes. We found that Gluckmann's technic gave no better differentiation of the argentaffin cells than the longer original Masson method.

[5]Gluckmann, F. 1947. La détection rapide des cellules chromaffines intestinales par les complexes ammoniacaux d'argent à chaud. C. R. Acad. Sci., **224**, 295.

WHOLE MOUNTS OF FLAT AND ROUND WORMS FOR MORPHOLOGICAL STUDIES

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Abstract.—**A method for making permanent whole mounts of flat and round worms is described. The specimens are mounted in a drop of acid fuchsin lacto-phenol on a slide and warmed for 6 hours at 60°C. The acid fuchsin is replaced by light cotton-blue (anilin blue, W. S.) in lacto-phenol, till the desired contrast is obtained. After this, the forms are mounted in pure lacto-phenol, using the coverglass. The margin of the coverglass is sealed with the sealing media devised by Dade and Waller (equal parts of damar balsam and beeswax).**

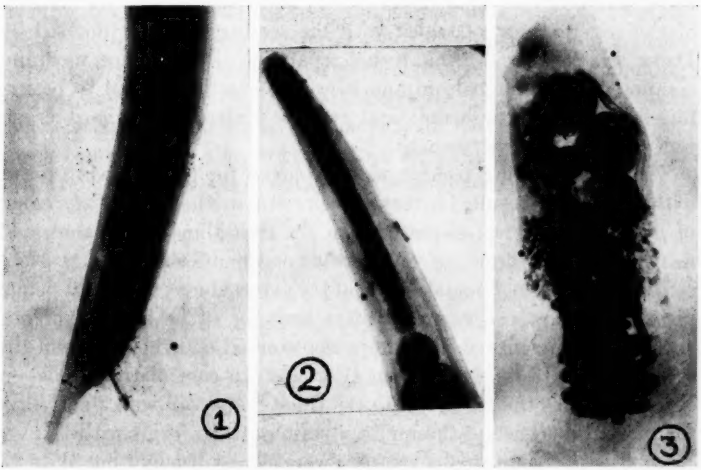

Fig. 1.—Nematode from the rectum of Pigeon. × 65. Penial setae are clearly seen.

Fig. 2.—Nematode from the rectum of frog. × 64. Anterior end showing the pharyngeal bulb.

Fig. 3.—Trematode from the rectum of frog. × 61. The uterus with eggs, the testes, vitellarium and the genital aperture are clearly seen.

In the course of studies on some nematode parasites of plants, the need for staining and clearing whole mounts for morphological study

was keenly felt. It was also desirable to make such slides permanent. The usual methods of staining and dehydrating did not give the desired result on account of shrinkage.

The following method was therefore adopted for staining and clearing the material, a process which has been used by Dr. M. J. Thirumalchar for staining fungus material. The specimens were mounted in a drop of water on a slide. The water was drained off and a drop of acid fuchsin in lacto-phenol added (acid fuchsin added to lacto-phenol till a cherry red color was obtained). The slide was then warmed on a hot plate to about 60°C. for nearly six hours. After this, the acid fuchsin lacto-phenol was drained off, a dilute solution of cotton-blue (anilin blue W. S.) in lacto-phenol was added and the slide was slightly warmed. Observation revealed that the acid fuchsin was replaced by cotton-blue in some of the tissues while being retained in others. When the desired amount of differentiation was obtained, the material was mounted in pure lacto-phenol using a coverglass. For making the slides permanent, the margins of the coverglass were sealed with the sealing medium (devised by Dade and Waller[1]) using a hot spatula. The sealing medium, composed of damar balsam and beeswax, has been found to be immiscible with lacto-phenol, and soon solidifies into a hard crust, rendering the slide permanent.

The same staining technic was employed for the following forms with very good results; nematodes present in the alimentary canal of frogs and Calotes; trematodes in the frog alimentary canal, and fish liver; tape-worms present in the pigeon intestine. It was observed in the case of nematodes that the external apertures and penial setae (Fig. 1) were very clear after staining. The internal organs such as the alimentary canal were also stained and the details of the structures could be clearly seen (Fig. 2). In case of trematodes, it was found that the forms with very thick body wall were poorly differentiated, because of the uniform staining. In forms with thinner body wall all the internal structures were clearly brought out (Fig. 3). The same method was also tried on plankton forms previously fixed in formalin. It was found that the acid fuchsin lacto-phenol method gave very good results and deserves a trial on similar forms.

In this connection I wish to thank Dr. L. S. Ramaswami, Professor of Zoology for his kind encouragement and also Dr. M. J. Thirumalchar for his kind help.

[1]Dade, H. A., and Waller, S. Mycol. Pap. Commonw. Inst., 27. 1949.

STAINING OF PLANT MATERIALS CLEARED IN NaOH[1]

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ABSTRACT.—**Stains are listed which have proved suitable for staining the epidermis, mesophyll, and sclerenchyma and tracheary elements, respectively, of cleared leaf material of *Mouriri* and *Linociera*. Too rapid leaching is avoided by overstaining high in the dehydration series, destaining briefly in the same solvent, and moving through to xylene. Twenty to thirty minutes staining time is generally sufficient. Concentrations and solvents can be varied widely. If destained too much, the material can usually be replaced in the dye with no ill effects. A double stain schedule (Bonnett) of five to ten minutes in 1% Bismarck brown Y in 95% alcohol followed by one to two minutes in 1% fast green FCF in 100% alcohol may be advantageous for thin-walled cells in thick material. It may be preferable to treat thinner material with tannic-acid-iron-chloride followed by safranin (Foster). The effects of bleaches and clearing compounds other than NaOH on staining have not been investigated; however, Dr. Bonnett finds that lactic acid used after NaOH improves clearing and also improves the staining of his combination (above). Mordants can doubtless be used to advantage.**

One of the simplest and best means of preparing plant material for the three-dimensional study of cell size, shape, and arrangement is that of clearing, done by bleaching or removing the pigments with suitable reagents. Although this method has long been known and used, its possibilities are not yet all exploited.

Cleared objects are often thin and transparent enough to be satisfactorily viewed when immersed in water or alcohol. In these media small veins and other details are usually readily visible, and no stains are needed. Frequently, however, the material is so opaque even after clearing that it must be run up into xylene for sufficient transparency. When this is done the increased refractive index of the medium renders many details of structure almost invisible, and a stain becomes desirable. Staining may also be needed to bring out thin-walled cells which are not easily seen even when in water.

Prolonged exposure to NaOH with the accompanying removal of many pigments and other substances alters the chemistry of the

[1]For an account of this clearing method, see Foster (1949, p. 216–217).

plant tissues. The result is that many structures no longer have the affinities they once had for certain dyes, and most dyes tend to leach out rapidly when in water or alcohol. Thus stains cannot be relied upon to act as they would on uncleared material. This is apparently true to a lesser extent of materials cleared in other reagents. particularly those which are simply bleaching compounds. Evidently little exploration of this general subject has been done, although Stevens (1916) and Gabriel and Pellisier (1936) each give instructions for the use of two dyes with cleared material, and Stebbins (1938) does likewise for a single stain.

Using whole leaf-material of *Mouriri* (Melastomaceae) and *Linociera* (Oleaceae) cleared in NaOH, over 20 different stains were tried in an effort to determine which could be used for bringing out the epidermis, mesophyll, and sclerenchyma and tracheary elements, respectively. The best of these are listed below, in approximate order of their successfulness.

Epidermis

1. $ZnCl_2$-tannic-acid-iron-alum. Sometimes more effective without the $ZnCl_2$; also when following treatment with Stockwell's fluid (see Johansen, 1940, p. 85). Use 1% tannic acid; otherwise follow Scharman (1943).
2. Harris' hematoxylin, stained to taste, blued with tap water; not destained.
3. Delafield's hematoxylin, *ca.* 20% aqueous. As with Harris'.
4. Aniline blue, *ca.* 1% in 95% ethyl alcohol. (Certification No. NK4.)
5. Safranin O, *ca.* 0.5% in half xylene, half 100% alcohol. (Certification No. NS–11.)
6. Methylene blue (chloride), *ca.* 0.5% in half xylene, half 100% alcohol. (Certification No. NA–12.)
7. Other general stains may serve.

Mesophyll (General Stains)

1. Foster's tannic-acid-iron-chloride (see Johansen, 1940, p. 91).
2. Fast green FCF, *ca.* 0.5% in 100% alcohol. (Certification No. NGf-6)
3. Congo red, *ca.* 1% aqueous or weak alcohol. Grübler.
4. Bismarck brown Y, *ca.* 1% in 100% alcohol. (Sample purchased from University Apparatus Co., Berkeley, Calif.)
5. Aniline blue, *ca.* 1% in 95% alcohol. (Certification No. NK-4 used)

6. Brilliant green, *ca.* 0.5% in 100% alcohol. (Certification No. NBg-13.)
7. Light green SF, *ca.* 0.5% in 100% alcohol. (Certification No. NL-9.)
8. Erythrosin, *ca.* 1% in 100% alcohol. (Harmer Laboratories Co.)

Sclerenchyma and Tracheary Elements

1. Safranin O, *ca.* 0.5% in half xylene, half 100% alcohol. (Certification No. NS-11.)
2. Methylene blue (chloride), *ca.* 0.5% in half xylene, half 100% alcohol. (Certification No. NA-12.)
3. Acid fuchsin, *ca.* 0.5% in 70% alcohol. (Certification No. NR-19.)
4. Crystal violet, *ca.* 0.5% in 100% alcohol. (Certification No. NC-21.)
5. Delafield's hematoxylin, overstained and de-stained in acid alcohol, 20% to 50% aqueous.
6. Bismarck brown Y, *ca.* 1% in 100% alcohol. (Sample purchased from University Apparatus Co., Berkeley, Calif.)
7. Iodine green, *ca.* 1% in 100% alcohol.

As can be seen, many of the above dyes stain approximately the same structures that they do in uncleared material, although usually not so well. The epidermis in particular stains poorly. It is possible that some of the microchemical tests may prove useful as stains, although few were tried by the writer.

In general, difficulties caused by rapid leaching are surmounted by overstaining high up in the dehydration series, destaining briefly in the same solvent, and moving through to xylene. If the dye is in water solution, the material must be run through as rapidly as possible. Twenty to thirty minutes staining is generally sufficient staining time for all the dyes listed, depending of course on the thickness of the material. The concentrations and solvents given can be varied widely. Destaining is often complete in fifteen minutes, although fast green, Congo red, acid fuchsin, and aniline blue tend to leach out more slowly than the rest. Rapidity of destaining and degree of differentiation depend on the material used; some dyes may not destain at all in certain cases. For most of the stains, removal can be accelerated with acidified alcohol. This includes tannic-acid-iron-chloride. If destained too much, the material can usually be replaced in the dye with no ill effects. Delafield's and Harris' hematoxylin require the use of acidified alcohol for destaining, but the stain is removed first from the cuticle and epidermis. Therefore these

regions will not show well if destaining is carried out. The Congo red can be improved by staining in about a 0.2% aqueous solution, moving to 95% alcohol, acidifying slightly with HCl until the dye turns dark purplish black, rinsing in 100% alcohol, and moving on up the series. Variability is great; staining qualities of leaves from different species and of leaves from different plants of the same species may be entirely different, and correct staining therefore becomes a matter of individual treatment.

Double staining is often advantageous. Stevens (1916, p. 302) recommended the use of Bismarck brown preceded by safranin. Two other workers have kindly permitted me to mention their procedures. Dr. O. T. Bonnett of the University of Illinois, working with cleared primordia of corn ears and tassels, has found a combination of Bismarck brown Y and fast green FCF most suitable for bringing out the thin-walled cells of the meristematic region and at the same time maintaining the high transparency needed with his thick material. He stains the material five to ten minutes in a 1% solution of Bismarck brown in 95% ethyl alcohol, destains in the same strength alcohol, stains one to two minutes in a 1% solution of fast green in 100% alcohol, and then if necessary gives the material a second treatment in a 1% solution of Bismarck brown in 100% alcohol to replace what dye the surface tissues may have lost. Dr. A. S. Foster, of this institution, investigating cleared leaves of *Quiina* (Quiinaceae), is successfully using tannic acid-iron chloride for a general stain, followed by safranin to give a contrasting background and to bring out the procambium and tracheary elements. Other combinations may likewise prove valuable.

Brownish pigments remain after clearing some materials, and must be bleached out. The writer, using Stockwell's fluid for this purpose (Johansen, 1940, p. 85), found that the subsequent staining was sometimes unaffected, sometimes improved, and other times was made unsatisfactory. The subject of bleaches, their effects, and the use of compounds to restore staining capacity, needs study.

Different clearing compounds may also affect staining, although the general uses of the different dyes will probably remain the same. For instance, material bleached in Chlorox and stained with safranin takes a more general stain than material cleared in NaOH, destains much more slowly, and at the same time differentiates the vascular elements more poorly. For various reasons the chloral hydrate (Stevens, 1916, pp. 299, 301), lactophenol (Klebahn, 1910), lactic acid (Simpson, 1929; Sporne, 1948), hydrogen peroxide with ammonium hydroxide (Stebbins, 1938; Sporne, 1948), sodium hypochlorite (McVeigh, 1935; Gabriel and Pellisier, 1936), and potassium hypo-

chlorite (Eau de Javelle: Stevens, 1916, p. 307) methods proved unsatisfactory for the material used here, and therefore the writer has only limited experience with them. Dr. Bonnett, however, has found that while lactic acid itself will not sufficiently clear his material, it improves clearing when used after NaOH, and at the same time improves the staining of his combination of Bismarck brown with fast green. Other stains as well may be made more effective. This leads to the whole question of mordants, another field which has not been explored with regard to cleared material. The writer had some success with iodine as a mordant for crystal violet and with Delafield's hematoxylin as one for methyl green, and it is probable that further use of mordants will yield worthwhile improvements in staining.

REFERENCES

FOSTER, ADRIANCE S. 1949. Practical Plant Anatomy. D. Van Nostrand Co.

GABRIEL, C. and PELLISIER, F. 1936. Une methode d' éclaircissement et d' étude d' anatomie végétale par transparence (*in situ*). Bull. Soc. Bot. France, **83**, 816–19.

JOHANSEN, DONALD A. 1940. Plant Microtechnique. McGraw-Hill, New York.

KLEBAHN, H. 1910. Krankheiten des Selleries. Z. Pflkrankh., **20**, I.

MCVEIGH, ILDA. 1935. A simple method for bleaching leaves. Stain Techn., **10**. 33–4.

SCHARMAN, B. C. 1943. Tannic acid and iron alum with safranin and orange G. Stain Techn. **18**: 105–111.

SIMPSON, JENNIE L. S. 1929. A short method of clearing plant tissues for anatomical studies. Stain Techn., **4**, 131–2.

SPORNE, KENNETH R. 1948. A note on a rapid clearing technique of wide application. New Phyt., **47**, 290–1.

STEBBINS, G. LEDYARD JR. 1938. A bleaching and clearing method for plant tissues. Science, **87**, 21–2.

STEVENS, WILLIAM C. 1916. Plant Anatomy and Handbook of Micro-Technic. Blakiston's Son and Co., Philadelphia.

DETERIORATION OF SCHIFF'S REAGENT

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ABSTRACT.—**Deterioration of Schiff's (Feulgen's) reagent was studied under varying conditions. Photometric readings were taken of both induced and spontaneous color development and curves which showed the rate of deterioration were plotted. It was concluded that Schiff's reagent, when kept in well-filled and tightly corked bottles at 0°–5°C., will retain staining efficiency for at least six months.**

This study was undertaken to observe accurately the deterioration of Schiff's (Feulgen's) reagent under varying conditions of time and temperature.

Essentially two factors are involved in its deterioration. One is an irreversible color change caused by exposure to air, and the other is a partly reversible change, caused by a shifting of chemical equilibrium between the sulfite and dye (Karrer, 1946). Fresh Schiff's reagent sealed in air-tight tubes will, upon exposure to temperatures of 60°C., rapidly take on a deep violet color. If the tubes are then cooled, the color disappears. This reversibility is still demonstrable after 24 hours at 60°C. After approximately 48 hours at 60°C., however, the coloration by heat tends to become permanent and not completely removed by cooling. Both the irreversible (oxidative) and the partially reversible (heat) changes in the reagent were observed under controlled conditions.

Schiff's reagent was prepared (de Tomasi, 1936) by dissolving 0.5 g. of basic fuchsin by pouring over it 100 ml. of boiling distilled water. The mixture was shaken thoroughly, allowed to cool to 50° C., and filtered; 10 ml. of 1*N* HCl followed by 0.5 g. of potassium metabisulfite ($K_2S_2O_5$) were added to the filtrate. The solution was allowed to stand in the dark, well stoppered, overnight. Residual color was removed (Coleman, 1938) by adding 0.25 g. of activated charcoal, shaking and filtering.

Equal amounts of solution were placed in glass stoppered bottles and stored at temperatures of 60°, 40°, 25°, 15° and 0° C. The ability of these solutions to develop color was checked weekly by

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means of a photometer. One-half milliliter of reagent, 1 ml. of 10% formalin, and 20 ml. of distilled water were placed in a test tube, and the color which developed in one-half hour was read photometrically against a distilled water standard, using a red (650 mμ) filter. The results are shown in Fig. 1. A photometric reading of 2 indicates

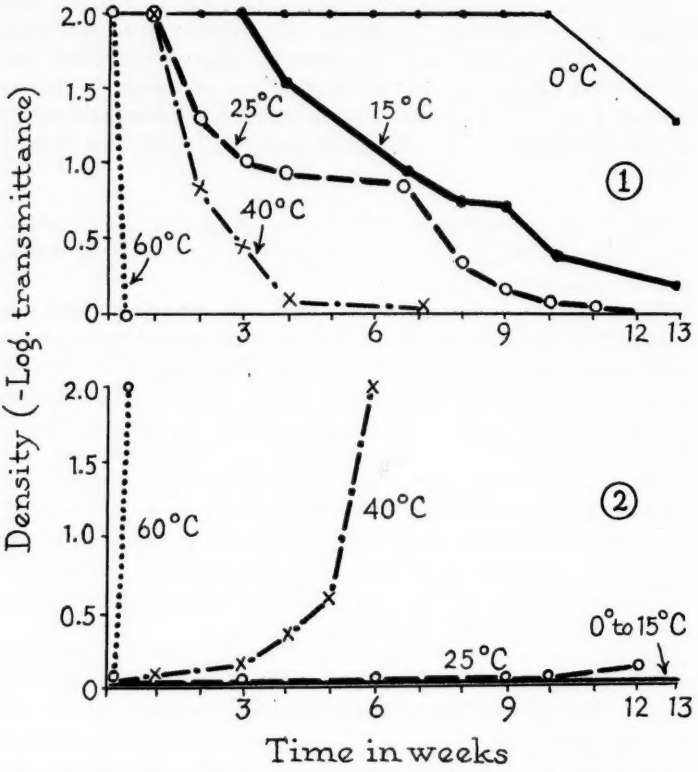

FIG. 1.—Amount of color developed by Schiff's reagent when formaldehyde was added after varying periods of storage at various temperatures.

FIG. 2.—Amount of color developed spontaneously by Schiff's reagent on storage at various temperatures.

the maximum color development possible by freshly prepared Schiff's reagent in an excess of formalin. The Coleman charcoal method was used to remove any recolorized dye that appeared spontaneously through the process of deterioration before taking the readings.

The curves in Fig. 1 show that deterioration is very rapid at 60° or even 40°, but slow between 0°–5°. This supports Lillie's (1947) suggestion that Schiff's reagent should be stored at temperatures from 0° to 5°.

At intervals, tissues were stained with the solutions by the Picro-Feulgen block method (Lhotka and Davenport, 1947) to determine the histological efficiency of the reagents. A photometric reading 0.669 coincided with the first histological evidence of deterioration in the solutions, and any reagent that had deteriorated to this point was considered to be unsatisfactory for staining.

The reversible, spontaneous production of color by heat was studied by placing 20 ml. of reagent in sealed test tubes. These tubes were stored at the same temperatures as the bottles. They were checked weekly for spontaneous color development by reading them photometrically in the same way that the formalin-developed color was read. No formalin was added to these tubes, and they remained sealed throughout the experiment. The findings are shown in Fig. 2. These curves show that heat deterioration is rapid at 60° but negligible below 25°. The initial reversibility of this reaction was shown, in that solutions at 60° which had developed a photometric reading of .824 were returned to a colorless state by cooling below 25°.

From these studies, it can be concluded that Schiff's reagent may be prepared for the Feulgen stain in stock quantities provided it is stored at temperatures of 0° to 5°C. and is contained in well filled, tightly stoppered bottles. The curves which show the course of its deterioration indicate that it will retain its staining efficiency for at least six months and probably longer.

ACKNOWLEDGMENT

The authors wish to express their appreciation of assistance received through a research grant from the Biological Stain Commission.

REFERENCES

COLEMAN, L. D. 1938. Preparation of leuco basic fuchsin for use in the Feulgen reaction. Stain Techn., **13**, 123–4.

KARRER, P. 1946. Organic Chemistry, Second English Edition. Elseveir Publishing Company, N. Y.

LHOTKA, J., and DAVENPORT, H. A. 1947. Differential staining of tissue in the block with picric acid and the Feulgen reaction. Stain Techn., **22**, 139–44.

LILLIE, R. D. 1947. Studies on the preservation and histologic demonstration of glycogen. Bul. Internat. Assoc. Med. Mus., **27**, 23–61.

SCHIFF, UGO. 1866. Eine neue Reihe organischer Diamine. Liebig's Annalen der Chemie und Pharmacie, **140**, 92–137.

DE TOMASI, J. A. 1936. Improving the technic of the Feulgen stain. Stain Techn., **11**, 137–44.

MOUNTING MEDIA FOR PHASE MICROSCOPE SPECIMENS

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ABSTRACT.—**Results with phase microscopy can often be improved by the proper selection of mounting media. Temporary mounts can first be made in liquid mixtures of water and glycerol, butyl carbitol and alpha-chloronaphthalene, alpha-chloronaphthalene and alpha-bromonaphthalene or alpha-bromonaphthalene with methylene iodide to determine what index liquid serves best to emphasize structures of primary interest. Once this has been determined, permanent mounts can be prepared by the substitution of a solid mounting medium that most closely approaches the refractive index of the liquid.**

Many papers have been published with reference to the superiority of phase microscopy over the usual brightfield and dark-field methods for the examination of living zoological and botanical specimens. Applied to paraffin sections of tissue, whole mounts or sections of textile fibers, and chemicals and minerals, phase microscopy has not always produced spectacular results, and in a few cases bright-field illumination has even been preferred. Barer (1947) states that for fixed neurological material phase microscopy is sometimes at a disadvantage. Too much detail is often rendered visible when a selective effect is desired, as is possible with the use of specific stains. Some of these apparent failures are due to the use of specimens that are too thick, incorrect cover-glass thickness when using the 43× (4 mm.) 0.65 N. A. objective, and the employment of mounting media of unsuitable refractive index. The choice of index liquids or solids for mounting is somewhat dependent on the relative refractive index of constituents within the preparation. For optimum results, objects of primary interest within the specimen should appear dark against a lighter background. This effect with positive contrast objectives can be obtained when the object of chief interest has a slightly higher refractive index than its background and the mounting medium employed is slightly below the object of interest in refractive index. It is the purpose of this paper to suggest a series of liquids and solid mounting media, some of which have been employed in brightfield and dark-field microscopy, that might be used to improve results with phase microscopy.

Mounting Media for Living Preparations

The primary characteristic of mounting media for living preparations is that they must not cause plasmolysis or plasmoptysis of the cell. Such isotonic media are either the liquids in which the cell lives or are artificially prepared solutions. Specific examples are protozoa or bacteria mounted in their culture media, blood cells in their serum or normal saline, and epithelial cells of the mouth in saliva. In all cases the mounting media consist mainly of water having an index of $n_D = 1.33$ or slightly higher, dependent on the amount of material in solution. A survey of the literature reveals that little information is available as to the refractive index of the living cell. Lee (1937) makes the statement that its index is slightly higher than water. Maximow and Bloom (1930) give the index of the lens of the eye as 1.36 in the peripheral layers and 1.42 in the inner zone or nucleus, where the consistency of the tissue is harder. From this information it would seem that these isotonic liquids for the living cell, being slightly below the index of most cell constituents, would be quite ideal for producing good contrast. In general this has proved to be true. For example, if an epithelial cell from the mouth is examined in saliva ($n_D = 1.33+$) with positive contrast phase objectives, the nucleus and most of the cytoplasmic granules, having a slightly higher index than the saliva, appear dark against the lighter background of the ground substance. The results for some structures within this preparation and for certain constituents of other living histological specimens might possibly be improved by the substitution of liquids of slightly higher index as mounting media. The difficulty involved in preparing any such liquids is to find an aqueous solution that has a higher index and at the same time is isotonic.

Mounting Media for Non-living Preparations

With reference to non-living preparations we are not limited to isotonic solutions and thus a larger number of mounting media are available. The question, however, is raised as to what index liquid or solid will provide the maximum contrast for certain structures within a textile fiber, inclusions within a crystal, mitochondria within a cell, chromatin in the nucleus, etc. This can be determined by the preparation of a series of index liquids as suggested for the dispersion staining method (Crossmon, 1948, 1949) ranging in index from about 1.33 to 1.70 and differing in index by 0.004. The specimen is temporarily mounted in these media to determine what index will give the greatest contrast to the object of primary interest. For an

index range of 1.33 to 1.436, mixtures of distilled water with glycerol are suggested; for 1.440 to 1.628, diethylene glycol monobutyl ether (butyl carbitol) and alpha-chloronaphthalene; for 1.632 to 1.656, alpha-chloronaphthalene and alpha-bromonaphthalene; and for 1.660 to 1.70, alpha-bromonaphthalene with methylene iodide. The quantity of the lower index liquid to be mixed with the higher to give the intermediate indices can be determined by the formula:

$$V_1n_1+V_2n_2=V_xn_x$$

where V represents volume, n the index and V_xn_x the volume and index desired. Before substitution of the index value for the lower and higher constituents in the formula, it is best to accurately determine their index by means of a refractometer at 25°C. To obtain 10 ml. of a 1.520 index liquid as is used as a mounting medium for the section of prostate gland, a mixture of 5.3 ml. of butyl carbitol with 4.7 ml. of alpha-chloronaphthalene would be required, as shown in the computation below:

$$\begin{aligned}
1.430V_1+1.620V_2&=10(1.520)\\
V_1+\quad V_2&=10 \text{ ml.}\\
1.430V_1+1.620V_2&=15.20\\
1.430V_1+1.430V_2&=14.30\\
\hline
0.190V_2&=0.90\\
V_2&=4.7 \text{ ml.}\\
V_1&=5.3 \text{ ml.}
\end{aligned}$$

For more accurate results than determined by the formula, the index of the liquids thus prepared should be checked on a refractometer at 25°C. and corrections made by adding the necessary quantity of the lower or higher index liquid.

The refractive indices of minerals, chemicals, textile fibers and plastics can be found in chemical handbooks or published papers, and from that information an idea can be formed as to the range of index liquids that should be used as mounting media. Little information is available concerning the refractive indices of fixed histological specimens. Our work with paraffin sections of formaldehyde-fixed tissue of the white rat indicates that many histological structures are slightly below or above 1.536. According to Manly (1938), dental structures are considerably higher. In a review of the literature, he finds that enamel has been reported as having an index of 1.627 for the ordinary ray and 1.6234 for the extraordinary ray. The index of normal dentine is given as 1.577 ± 0.003. Secondary cementum, according to his own investigation, varies in index from 1.560 to 1.570.

The procedure for temporary mounting of histological preparations is as follows. Paraffin microtome sections of suitable thickness (6–10 μ) of zoological or botanical material or of textile fibers should be attached to clean slides by the water and desiccation method (Crossmon, 1948) and after drying placed in xylene to remove the paraffin. In preparation for mounting, the xylene surrounding the section should be wiped away with a lint-free cloth and the section gently blotted face down against blotting paper. A drop of one of the index liquids (about 1.520 is suggested as a trial for formaldehyde-fixed tissue) is placed on the preparation and a cover-glass applied. The thickness of the cover-glass used is very important when using the 43×(4 mm.) 0.65 N.A. objective. Best results are obtained when its thickness is close to 0.18 mm. Should a preparation as a whole or constitutents within the preparation appear white, the index liquid used is either much lower or higher than the object. An illustration is to first mount a microtome section in water ($n_D = 1.33$) and after dehydrating remount in methylene iodide ($n_D = 1.74$). In both cases all cell constituents will appear white with little or no detail. However, as we substitute liquids closer to the index of the specimen, structures slightly above the liquid will appear dark and much greater contrast will be evident.

The index liquids suggested are only temporary mounts. For permanent preparations the cover-glass must be ringed with cement or solid mounting media must be substituted. Let us suppose that a certain structure in a neurological preparation gives the best contrast in a 1.534 liquid and that we wish to have a permanent preparation. A reference to the list of solid mounting media given in this paper gives the index of solid Canada balsam as about 1.53. This should prove to be a suitable substitute. Consideration must be made that its solvent such as xylene lowers its index and it is not until the solvent has evaporated that the index of solid balsam is attained. Also, balsam, as well as other natural resins, varies slightly in index dependent on the method of preparation.

Other solid mounting media that have been used in microscopical technic or have been suggested in the literature are given in the table below. Detailed information concerning their preparation, properties or source can be found in the references named. The refractive indices given are as stated in the literature cited. They should be accepted with some caution, since for the most part no information is given as to whether the index stated represents the solid or solid dissolved in a solvent.

The photographs of unstained prostate gland reproduced in this publication are typical of the different contrasts that can be obtained

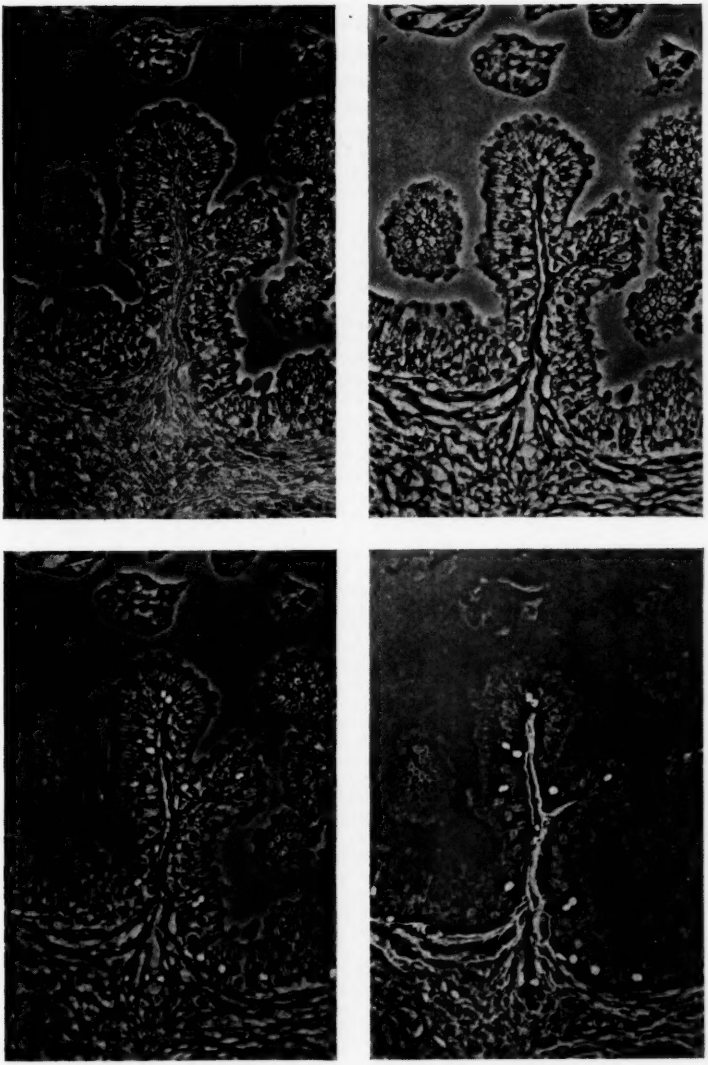

FIG. 1–4.—All photographs are of exactly the same area of a 10μ section of human prostate gland fixed in formalin, the only difference being the index of the mounting media (butyl carbitol with alpha-chloronaphthalene). Mag. 300× using the 21× (8 mm.) 0.50 N.A. objective.

FIG. 1.—Refractive index of mounting medium n_D 1.430.
FIG. 2.—Refractive index of mounting medium n_D 1.508.
FIG. 3.—Refractive index of mounting medium n_D 1.520.
FIG. 4.—Refractive index of mounting medium n_D 1.548

dependent on the mounting medium employed. Fig. 1 shows a section mounted in a liquid of n_D 1.430, considerably lower in index than the majority of the tissue constituents. Most of the tissue elements are white and little contrast is evident. The mounting media employed in Fig. 2 and 3 are both almost ideal dependent on what

TABLE 1. REFRACTIVE INDEX OF VARIOUS MOUNTING MEDIA

Name	Refractive Index	Reference
Glychrogel A	1.41 (solid)	Stain Techn., **10**, 21–2. 1935.
Isobutyl methacrylate	1.47	Shillaber, C. P. Photomicrography, p. 498. 1944.
Glycerin jelly	1.48	Shillaber, C. P. Photomicrography, p. 497. 1944.
Sandarac camphloral	1.485	Lee, A. B. The Microtomist's Vade-Mecum, p. 228. 1937.
Camsal balsam	1.485	Lee, A. B. The Microtomist's Vade-Mecum, p. 227. 1937.
Aroclor and Isobutyl methacrylate	1.495–1.525 (solid)	Text. Res. J., **18**, 756–8. 1948.
Dammar	1.52	Chamot, E. M., and Mason, C. W. Handbook of Chemical Microscopy, p. 167. 1938.
Canada balsam	1.53 (solid)	Chamot, E. M., and Mason, C. W. Handbook of Chemical Microscopy, p. 167. 1938.
Euparal	1.53 (solid)	Chamot, E. M., and Mason, C. W. Handbook of Chemical Microscopy, p. 167. 1938.
Clarite	1.54 (solid)	Shillaber, C. P. Photomicrography, p. 502. 1944.
Clarite X	1.57 (solid)	Shillaber, C. P. Photomicrography, p. 502. 1944.
European styrax	1.582	Corrington, J. D. Working with the Microscope, p. 207. 1941.
American styrax	1.63	Corrington, J. D. Working with the Microscope, p. 207. 1941.
Aroclor	1.63	Shillaber, C. P. Photomicrography, p. 503. 1944.
Styrene resin	1.66	Chamot, E. M., and Mason, C. W. Handbook of Chemical Microscopy, p. 167. 1938.
Piperine	1.681	Corrington, J. D. Working with the Microscope, p. 207. 1941.
Hyrax	1.71	Corrington, J. D. Working with the Microscope, p. 207. 1941.
Glychrogel B	1.75 (solid)	Stain Techn., **10**, 21–2. 1935.
Pleurax	1.75	J. Roy. Micr. Soc., **69**, 25–8. 1949.
Naphrax	1.76	J. Roy. Micr. Soc., **63**, 34–7. 1943.
Sirax	1.8	Corrington, J. D. Working with the Microscope, p. 207. 1941.

tissue structures are of primary interest. Note especially the dark connective tissue in Fig. 2 and the white structures (probably nuclei of much lower or higher refractive index than the mounting medium) in Fig. 3. Fig. 4 represents the effect obtained when mounting medium of too high an index is employed. Note that the connective tissue shown as dark in Fig. 2 is light in this photograph.

Acknowledgment

The writer wishes to thank Mr. Louis Schrader for his assistance in preparing the photographs and index liquids necessary for the completion of this work.

REFERENCES

Barer, R. 1947. Some applications of phase-contrast microscopy. Quart. J. Micr. Sci., **88**, 491–500.

Crossmon, G. C. 1948. Optical staining of tissue. J. Opt. Soc. Amer., **38**, 417.

Crossmon, G. C. 1948. Microscopical distinction of corundum among its natural and artificial associates. Anal. Chem., **20**, 967–77.

Crossmon, G. C. 1948. The dispersion staining method for the selective coloration of tissue. Stain Techn., **24**, 61–5.

Crossmon, G. C. 1949. Dispersion staining. Educ. Focus, **20**, 4–10.

Lee, A. B. 1937. The Microtomist's Vade-Mecum, *10th Ed.* P. Blakiston's Son and Co., Philadelphia, Pa.

Manly, R. S. 1938. Relation of refractive index to density in dental hard tissues J. Amer. Chem. Soc., **60**, 2884–6.

Maximow, A. A., and Bloom, W. 1930. A Textbook of Histology. W. B. Saunders Co., Philadelphia, Pa.

NOTES ON TECHNIC

A Suggestion for Prevention of Loose Sections in the Bodian Protargol Method

Prolonged immersion in an aqueous medium such as the protargol bath in the original Bodian method for paraffin sections may increase any tendency for sections to separate from the slide during their subsequent reduction.

Even though all usual precautions against loosening have been taken we find that losses from detached sections are often suffered, particularly if the object is large and of heterogeneous character (e.g. the kidney) and therefore difficult to flatten properly.

For those who still prefer the longer immersion time of the original method to shorter modifications the following simple suggestion should be helpful.

Acidifying the rinse which preceeds reduction in sodium sulfite and hydroquinone with as little as 0.5 ml. of 2% acetic acid to 50 ml. of water greatly reduces losses from sections which might otherwise loosen. We have tested this suggestion repeatedly for a period of several months and find that detached sections rarely result and that the differentiation of the staining is improved as well.—Helen Davies and Pinckney J. Harman, *Department of Anatomy, College of Medicine, New York University.*

The Differential Staining of Amphibian Yolk Granules

Sections of frog ova and embryos can be stained in an aqueous solution of thionin, and then treated with alcoholic eosin; as a result the yolk is differentiated into basophilic and eosinophilic granules. The procedure is as follows:

1. Specimens are fixed in Smith's modification of Tellyesnicky's fluid for 24 hours, washed in running water for six hours, and then passed through the alcohols (30%, 50%, 70%, 83%, and 95%), remaining in each for 20–30 minutes; fresh 95% alcohol for 30 minutes follows.

2. Clearing in cedarwood oil for two hours is followed by immersion in xylene for three to five minutes; treatment with melted paraffin (m.p. about 52°C.) for three to five hours follows, during which time the paraffin is changed once.

3. Embedding, sectioning, and affixation to slides is carried out in the usual manner.

4. Paraffin is removed from the sections by placing the slides in xylene for three minutes.

5. Immersion in absolute alcohol for one minute is followed by passage through the alcohols (95%, 70%, 50%, and 30%), allowing thirty seconds in each.

6. The slides are rinsed in water for fifteen seconds, then placed in a saturated aqueous solution of *thionin* (C.I. No. 920) for one to two hours. After this the slides are rapidly rinsed in water, and all excess water is carefully wiped off.

7. Material is immersed in 95% alcohol for 30 seconds, then stained with 0.5% alcoholic *Eosin Y* (C. I. No. 768) for 15–30 seconds.

8. Treatment with 95% alcohol for 30 seconds is followed by mounting in green Euparal.

This technic clearly differentiates the large vegetal yolk granules (blue) from the small granules (red) making up the remainder of the yolk. It may therefore be of value to investigations concerned with tracing the disposition and utilization of the yolk granules in various stages of amphibian development.

Stock solutions of the stains are prepared by dissolving 0.5 g. of eosin in 100 ml. of 95% alcohol, and by dissolving 0.25 g. of thionin in 100 ml. of distilled water; the stains used by the author were manufactured by the National Aniline Division, Allied Chemical and Dye Corp.—Ira J. Laufer, *New York University, New York, N. Y.*

LABORATORY HINTS
FROM THE LITERATURE

DEPARTMENT DEVOTED TO ABSTRACTS OF BOOKS AND PAPERS FROM OTHER JOURNALS DEALING WITH STAINS AND MICROSCOPIC TECHNIC IN GENERAL

MICROSCOPE AND OTHER APPARATUS

HILLIER, J. **Some remarks on the image contrast in electron microscopy and the two-component objective.** *J. Bact.*, **57**, 313–7. 1949.

The introduction of a weak lens component between the specimen and the conventional electron microscope lens has increased the working distance of the objective ten times though the focal length is essentially unchanged. Bacteria photographed with the new objective show considerably more internal structure than was revealed with the conventional objective. However, both spherical and chromatic aberrations are also increased.—*V. Kavanagh.*

DYES AND THEIR BIOLOGICAL USES

PACKARD, C. E. **The effects of certain chemicals on the macronucleus of Spirostomum teres, with notes on the genus.** *Trans. Amer. Micr. Soc.*, **67**, 275–9. 1948.

Safranin and methyl green in varying concentrations were used as vital stains. The macronuclei showed considerable variation in size and shape in different concentrations of these dyes.—*V. Kavanagh.*

ANIMAL MICROTECHNIC

ARCADI, J. A. **Hortega's silver carbonate method applied to gill parasites; with a note on the head organs.** *Trans. Amer. Micr. Soc.*, **67**, 285–9. 1948.

Silver technics seem never before to have been applied to gill flukes but gave exceptionally clear pictures. The method was applied as follows: Fix parasites freed of mucus in 1% formalin for 4 days; transfer the parasites with a capillary pipette to concentrated NH_4OH for 3–4 min.; wash thoroughly in 2 changes of distilled water; impregnate in the prepared ammoniacal silver solution for 30 sec.–8 min. (50 sec. optimum); wash in 2 changes distilled water; reduce in 1% formalin for 30 sec.; wash as above; fix in 1% $Na_2S_2O_3$ a few sec.; wash in distilled water; mount in glycerin-gelatin medium as modified by Mizelle and Seamster (J. Parasitol., **25**, 501–7, 1939). The ammoniacal silver solution which gave best results was prepared as follows: 5 ml. 10% $AgNO_3$, 20 ml. 5% Na_2CO_3, concentrated NH_4OH to dissolve the precipitate and distilled water to make 75 ml.—*V. Kavanagh.*

BRECHER, GEORGE. **The structure of unstained reticulocytes.** *Proc. Soc. Exp. Biol. and Med.*, **69**, 89–90. 1948.

Examination of a wet film of mouse blood with a phase microscope revealed an occasional red cell which contained a delicate reticulum. Adding hypotonic potassium or ammonium oxalate to the blood increased the number of cells which showed intracytoplasmic structures, particularly rods and granules. Smears that were dried and fixed in alcohol failed to show these cytoplasmic structures. Dried smears mounted in 10% formalin or 1.2% ammonium oxalate showed structures similar to those seen in the fresh preparations. The identity of the cells which contained rods and granules with reticulocytes could be demonstrated by staining with a small amount (concentration not stated) of brilliant cresyl blue or of new methylene blue added to the oxalate solution. Human reticulocytes contained rods and granules similar to those seen in the mouse.—*H. A. Davenport.*

FIGGE, FRANK H. J., WEILAND, G. S., and MANGANIELLO, L. O. J. **Cancer detection and therapy. Affinity of neoplastic, embryonic, and traumatized tissues for porphyrins and metalloporphyrins.** *Proc. Soc. Exp. Biol. and Med.*, **69**, 640–41. 1948.

Hematoporphyrin in 1 mg. doses was injected intraperitoneally into tumor

bearing and non-tumor-bearing mice. Sacrifice at 24-hour intervals showed that most of the porphyrin migrated to the tumors in 24–48 hr. Porphyrin was demonstrated by its fluorescence, and its presence in tissues other than tumors found to be limited to the great omentum and lymph nodes. Tumors of uninjected tumor bearing mice were negative for hematoporphyrin. Other porphyrins (protoporphyrin, mesoporphyrin and coproporphyrin) showed the same tendency to be concentrated in neoplastic tissue, but other fluorescent substances (fluorescein, rhodamine, dihydro-collidine and riboflavine) did not. A zinc-porphyrin compound was concentrated by tumors. The possibility of using radioactive porphyrin in the therapy of lymphatic leukemia and in the detection of deep cancer is suggested.—*H. A. Davenport.*

ISENBERG, H. D. **Simplified method for staining spermatozoa.** *Amer. J. Clin. Path.*, **18**, 94. 1949.

The following modification of Meaker's staining procedure is described as a simple method of obtaining clear pictures of spermatozoan morphology: Shake manually at least 5 minutes 1 part of seminal fluid with 1 part of 5% $NaHCO_3$; centrifuge 2–3 minutes at 2000–3000 R.P.M.; pour off supernatant; wash centrifugate in normal saline corresponding to original volume and recentrifuge; repeat saline washing; add a few drops of saline to centrifugate and prepare smear with loop as for bacterial stain; dry at room temperature or at 37°C. in incubator, and fix 2–3 sec. with heat; flush slide with 95% ethanol, drain and allow to dry; stain 3 min. with a 1:1 mixture of carbol-fuchsin (no details) and 95% ethanol; wash in water, and treat 2 min. with a 1:3 aqueous methylene blue solution (no details); rinse at least 1 min. in running water, dry without blotting. The spermatozoa head-caps stain light blue, the nuclear posterior portion of the heads dark blue, and the body and tail portions light red or pink.—*L. Farber.*

KITZMILLER, J. B. **The use of dioxane in insect microtechnique.** *Trans. Amer. Micr. Soc.*, **67**, 226–30. 1948.

The use of dioxane simplified the process of sectioning aphids. The method is as follows: Bouin's fluid, 1 hr. to several days; dioxane, 1 hr.; fresh dioxane, overnight; Tissuemat, 2 hr. to overnight; embed in 56–58° Tissuemat. The longer times were advantageous except for aphids in the first or second instars. The aphids were handled in shallow gauze-bottomed dishes made from 1 in. glass tubing cut in ¾ in. sections to which gauze was tied tightly. These dishes were set in Stender dishes of the reagents. Embedding was done in the sloping molds suggested by Solberg.—*V. Kavanagh.*

PLANT MICROTECHNIC

BROADFOOT, H. H., and SCHWARZ, E. R. **An improved permanent mounting medium for textile fibers.** *Textile Res. J.*, **18**, 756–8. 1948.

Permanent colorless mounting media for textile fibers can be prepared by mixing together a synthetic resin (isobutyl methacrylate, n_D 1.48) with a plasticizer, Aroclor 1242, n_D 1.62, and dissolving the mixture in xylene to a viscosity close to that of balsam. By varying the proportion of each constituent it is possible to adjust the refractive index to a value suitable for a particular fiber. Mixtures having a refractive index of between 1.495 and 1.520 are suggested for cotton and wool, and between 1.515 and 1.520 for acetate rayon. The procedure for mounting is essentially the same as for Canada balsam.—*G. Crossmon.*

MICROÖRGANISMS

LORD, THOMAS H., and SMULL, MARGARET M. **An improved microscopic method of examining fatty foods.** *Food Res.*, **14**, 241–2. 1949.

An improved method for making smears of fatty foods for microscopic examination for bacteria is outlined as follows: (a) Melt the sample of oleomargine at 45°C. (113°F.) within a 15-min. period. Shake thoroughly. (b) Place 0.1 ml. of the sample on a standard glass slide and add one drop of Mayer's solution and three drops of a 1% "Tide" solution. (c) Mix vigorously with an "L"-shaped inoculating needle and then smear over the entire area of the slide. (d) Fix quickly by passing the slide through the flame of the Bunsen burner three times This is done rapidly so that the oil droplets will not coalesce into large areas but

will remain minute and evenly distributed over the slide. (e) Drip xylene over the slide to remove the fat, then immerse the slide in 95% alcohol from 30 to 40 sec. Dry in air. (f) Again drip xylene over the smear and dry in the air. Repeat until the smear is "cleared"; then dry. (g) Stain with Loeffler's alkaline methylene blue stain for about 30 sec. (h) Wash by dipping the slide into two jars of water. Allow to dry at room temperature. (i) Examine under the oil immersion lens, the field of which has been standardized to a diameter of 0.157 mm. The average number of organisms in 100 fields multiplied by 1,000,000 equals the number of organisms per milliliter of melted sample.

The improved appearance of the finished smear and the greater consistency of results led the authors to submit this method for general use by others. Although "Tide" is not a pure chemical compound, its general availability and low cost made it seem a desirable ingredient to use, especially under field conditions. Any other surface-active agent or detergent should prove equally useful, provided it does not cause disruption of the cells or alter the staining properties of the smears.—*William. G. Walter.*

MAY, J. **Zur fluoreszenzmikroskopischen Unterscheidung lebender und toter Bakterien mittels Akridinorangefaerbung.** *Zentbl. Bakt., I Abt. Orig.*, **152**, 586–90. 1948.

Varied fluorescent-microscopical examinations of 18 different microörganisms, stained with acridine-orange were performed. This was done to test Strugger's claim that living and dead bacteria can be differentiated by their color reaction. The author disclaims the validity of Strugger's theory. He found that the green, orange, and red color reflex of bacteria depends on concentration and pH of the dye solutions. The color scheme does not allow differentiation between living and dead bacteria.—*Ivan Saphra.* (Courtesy *Biological Abstracts*).

MICHAEL, PAUL, and MARKELL, E. K. **Modified Papanicolaou stain for amebae.** *Amer. J. Clin. Path.*, **19**, 81. 1949.

A combination of the iron hematoxylin stain with the Papanicolaou stain used as a counterstain is reported to improve the accuracy in differentiating amebae from other cells, such as histiocytes, to enable cytopathologic changes to be demonstrated. The recommended technic is as follows: Prepare smears as usual; before they have become dry, fix for 30 min. in freshly prepared Schaudinn's fluid containing 5% glacial acetic acid; rinse thoroughly in 70% ethanol, and treat 5 min. with 95% ethanol containing enough Gram's iodine to produce a port wine color; rinse thoroughly in 70% and 50% ethanol and in tap water; mordant 4 min. in 4% ferric ammonium sulfate; rinse in tap water; stain 10 min. in 0.5% aqueous hematoxylin; rinse in tap water; destain 12 min. in 0.25% ferric ammonium sulfate (made fresh daily from 4% stock solution), wash 5 min. in running tap water and rinse in distilled water; rinse successively in 50, 70, and 95% ethanol; stain 3 min. in OG6; rinse in 2 changes of 95% ethanol; stain 6 min. in EA 50; rinse in 3 changes of 95% ethanol followed by absolute ethanol; transfer to xylene; and mount in balsam or clarite.—*L. Farber.*

NICHOLS, M. STARR. **Gentian (crystal) violet solution for Gram stain.** *Amer. J. Clin. Path.*, **18**, 98. 1949.

The recommended solution is prepared as follows: Dissolve 2 g. oxalic acid in 880 ml. of water, add 20 ml. of aniline and shake to dissolve; dissolve 5 g. gentian (crystal) violet in 100 ml. of 95% ethanol and mix with oxalic-acid-aniline solution. It is claimed that this solution stains Gram-positive organisms well, and not any of the Gram-negative ones so far tested, and that it has good keeping qualities without developing any precipitate.—*L. Farber.*

PENNINGTON, DERROL. **The use of periodate in microbiological staining.** *J. Bact.*, **57**, 163–7. 1949.

Polysaccharides may be demonstrated by a periodate oxidation to form aldehyde groups which may be demonstrated by Schiff's reagent. The method is as follows: Fix bacteria by heat, in Bouin's solution, or other fixative; immerse them in 1% aqueous solution of sodium metaperiodate for 5–15 min. at room temperature; wash thoroughly with water; stain 15 min. in sulfite-decolorized basic fuchsin (Coleman, Stain Techn., **13**, 123–4, 1938); wash 10 min. in SO_2

water (5 ml. 10% $K_2S_2O_5$ and 5 ml. 0.1 *N* HCl in 100 ml. water); wash in water; dry. Metachromatin or volutin granules in spirilla and diphtheria bacilli disintegrate with the periodate.—*V. Kavanagh.*

RAWSON, ARNOLD J. **Acid-fast property of Histoplasma capsulatum.** *Amer. J. Clin. Path.*, **18**, 97. 1949.

Tissue sections have been stained with the following modification of the Ziehl-Neelsen method with a larger number of organisms retaining the carbolfuchsin stain than with the usual technic. Instead of using acid alcohol, 3% aqueous hydrochloric acid is used to decolorize the stained sections, which are then stained 30 sec. with methylene blue and passed rapidly through 95% and absolute ethanol before being placed in xylene. As with other staining procedures, only a variable number of the forms present were acid-fast.—*L. Farber.*

WEINREBE, N. **Stain for Borrelia recurrentis.** *Amer. J. Clin. Path.*, **18**, 99. 1949.

It is claimed that the following modification of Giemsa's staining technic for *B. recurrentis* gives more distinct preparations than the usual one. The thick drop smear (as for malarial stain) is dried without fixation in methyl alcohol and stained 1 hour in a mixture of 2 drops Giemsa's stain and 1 ml. of distilled water.—*L. Farber.*

HISTOCHEMISTRY

BOYD, GEORGE A., and WILLIAMS, AGNES I. **Stripping film technics for histological autoradiographs.** *Proc. Soc. Exp. Biol. and Med.*, **69**, 225–32. 1948.

Nuclear track emulsion stripping film (Eastman Kodak Co. NTB) was used successfully for autoradiography of histological sections. Such film, with a 10 μ cellulose ester base and a 10 μ thickness of emulsion, permitted mounting the section on either the emulsion side or the cellulose-base side. A variation in the distance between the section and the emulsion was advantageous in that it facilitated the identification of the actual cell or other histologic structure which contained the radioactive tracer. By mounting the section on the cellulose-base side of the film, developing the emulsion could be accomplished without subjecting the section to deleterious actions of the photographic developing and fixing solutions. Illustrations are given of the alpha particle "sunbursts" and the exact histologic site from whence the particle came. Artifacts caused by accidental abrasion of the emulsion and by the process of stripping are discussed.—*H. A. Davenport.*

CARRERA, G. M., and CHANGUS, G. W. **Demonstration of acid phosphatase in *Endamoeba histolytica*.** *Proc. Soc. Exp. Biol. and Med.*, **68**, 610–11. 1948.

Acid phosphatase activity was shown by Gomori's method. Smears containing *E. histolytica* were allowed to dry at room temperature until they became "glossy" but not completely dry, and fixed by immersion in chilled acetone for 30 sec. A positive reaction was obtained, however, on smears stored 26 hr. in acetone in the refrigerator. Incubation in the reaction mixture at room temperature (26–29° C.) 45 min. to 2 hr. sufficed to give a deposit of phosphate in the amebae. Negative results were obtained when the glycerophosphate was omitted from the incubation mixture or if NaF (*M*/300) was added to it. Two figures illustrate the stained specimen and an unstained control.—*H. A. Davenport.*

DOYLE, WILLIAM L. **Effects of dehydrating agents on phosphatases in the lymphatic nodules of the rabbit appendix.** *Proc. Soc. Exp. Biol. and Med.*, **69**, 43–4. 1948.

The loss of activity of both acid and alkaline phosphatases was studied after the following methods of preservation: absolute acetone and 80% acetone, absolute and 80% ethyl alcohol, absolute methyl alcohol, and quick freezing followed by drying *in vacuo*. The activity of freshly homogenized tissue in terms of phenol liberated from disodium phenyl phosphate in a glycine or barbiturate buffer was optimum for alkaline phosphatase at pH 9.8 and was 7–11 μg. of phenol per milligram of fresh tissue during 30 min. at 30° C. Acid phosphatase was

most active at pH 5.6–5.8 and liberated 10–14 μg. of phenol under the same conditions of time and temperature. Alkaline phosphatase activity of fresh tissue was found to be reduced to about 75% of the fresh value after fixation for 24 hr. in either absolute acetone, 80% acetone or absolute alcohol. After 80% ethyl alcohol, the activity was found to be about 65%. Only about 15% remained after fixation in absolute methyl alcohol. Acid phosphatase showed 25% of its original activity after acetone, 15% after ethyl alcohol, and a negligible amount after methyl alcohol. Better cytological fixation and preservation of enzymes were obtained when the tissue was dropped into acetone at −20° C. and stored at this temperature for 48 hr. Acetone-fixed material came through paraffin embedding with 60% acid, and 55% alkaline phosphatase activity. Phosphatases were stable at room temperature for several days in the block but deteriorated in ribbons. However, ribbons stored at −20° C. kept their activity for a week.—*H. A. Davenport.*

GOMORI, G. **Histochemical demonstration of sites of choline esterase activity.** *Proc. Soc. Exp. Biol. and Med.*, **68**, 354–8. 1948.

The procedure recommended is as follows: Fix and dehydrate tissue in ice-cold acetone. Transfer to a 1:1 mixture of absolute alcohol and ether for 1–3 hr., then into a 4% solution of collodion in alcohol-ether for 12 hr. while still in the refrigerator. Pass through two 1-hour changes of chloroform at room temperature and embed in paraffin. Cut sections and mount as usual, remove paraffin and bring to distilled water. Incubate the slides in a buffered solution of choline ester for 2–16 hr. at 37° C. The buffer stock solution is made by dissolving 29 g. of maleic acid and 30.4 g. of tris (hydroxy-methyl)-aminomethane in 500 ml. of water, decolorizing with a few grams of charcoal and filtering. To 10 ml. of this stock solution, 12 ml. of 0.5*N* NaOH is added (ph 7.7) and the mixture diluted to 50 ml. The working solution is made by adding 30 ml. of 0.1*M* cobaltous acetate diluted to 300 ml. with distilled water to the pH 7.7 buffer and then adding about 1 mg. each of $CaCl_2$, $MgCl_2$ and $MnCl_2$. The choline ester is prepared as a 0.02*M* solution in distilled water. The ester solution is added to the completed buffer mixture in the proportion of 1 ml. ester to 50 ml. of buffer at 37–40° C. Myristoyl choline ester is recommended as the most satisfactory. Lauroyl, palmitoyl, and stearoyl choline esters were tried also. After incubation, the slides are washed in tap water and then immersed in a dilute solution of light yellow ammonium sulfide (a few drops of the concentrated reagent to 50 ml. of distilled water) for 15 min. They are then washed under the tap, counterstained as desired, dehydrated, cleared and covered. The sites of choline esterase activity are stained black. Five figures are given which show esterase activity in the nerve cells of the brain of the mouse, the conducting system of the heart in the dog, spindle structures in striped muscle of the mouse, and the reactions to two different substrates of the sympathetic ganglia of the rabbit. The reaction is inhibited in most tissues by 0.00001*M* prostigmine.—*H. A. Davenport.*

GOMORI, G. **Histochemical demonstration of sites of phosphamidase activity.** *Proc. Soc. Exp. Biol. and Med.*, **69**, 407–9. 1948.

In a study of the histochemical specificity of phosphatases (Gomori, Proc. Soc. Exp. Biol. and Med., **70**, 7–11, 1949—following abstract), one substrate out of 19 gave a reaction at pH 5 which indicated the existence of a specific enzyme, a "phosphamidase". The unique substrate, p-chloranilidophosphonic acid, was processed to yield the purified, free phosphonic acid, and this compound was used as a substrate with histologic sections to determine the sites of phosphamidase activity. The following procedure is recommended: Fix thin slices of tissue in chilled acetone, or in 95% or absolute alcohol, and make paraffin sections therefrom. Deparaffinize, bring to water, and incubate in substrate-buffer mixture. Prepare the buffered mixture by taking 50 ml. of 0.05 *M* maleate buffer, pH 5.6 (5.8 g. maleic acid plus 62 ml. of *N* NaOH in 1000 ml.), adding thereto 1.0–1.5 ml. of 0.1 *M* $Pb(NO_3)_2$ solution, a few drops of 10% solution of $MnCl_2$, and shaking until the precipitate dissolves. Add 2 ml. of the phosphonate stock solution made by dissolving a calculated amount of the purified phosphonic acid in an excess of 10% NH_4OH, adjusting to pH 8 with acetic acid, and bringing up to volume with distilled water. Place the staining mixture in an oven at 45–60° C. until the $Pb_3(PO_4)_2$ settles. Filter into a Coplin jar which is subsequently inclined at an angle of 30° and place the slides face downward in it.

After incubation for 10–24 hr. at 37–40°, rinse the slides in distilled water and wipe precipitate from the backs of the slides and around the tissue. Remove the remaining superficial precipitate by placing the slides in a 0.1 *M*. citrate buffer, pH 4.5–5.0, and rinse thoroughly under the tap as soon as the slide appears to be completely clear around the tissue. (This differentiation is the most critical step in the procedure since insufficient treatment may leave precipitate which appears black in the finished stain and overtreatment may remove the enzymatic reaction product.) Treat the slides with a dilute solution of yellow ammonium sulfide (about 10 drops of the concentrated reagent to the Coplin jar). Wash in tap water, counterstain if desired, dehydrate, clear and cover. Treatment for 10 min. in Lugol's solution or in any 1 *N* mineral acid inactivates the enzyme and permits the making of control slides. Small amounts of the enzyme are present in many normal tissues and large amounts are found in the gray matter of the central nervous system and in malignant epithelial tumors.—*H. A. Davenport.*

GOMORI, G. **Histochemical specificity of phosphatases.** *Proc. Soc. Exp. Biol. and Med.*, **70**, 7–11. 1949.

The problems of substrate specificity, organ specificity and effects of specific activators and inhibitors are discussed briefly. The experimental data were obtained by the application of histochemical technic. Six to 20 different tissues, fixed in chilled alcohol or acetone were embedded in a single paraffin block. Sections were mounted on slides and incubated 1–24 hr. with the substrate to be tested. The following substrates were used: metaphosphate; pyrophosphate; methyl-, glycero-, and aminoethyl phosphate; phenyl, o-chlorophenyl, *a*-naphthyl-, resorcyl- and phenolphthalein phosphate; glucose-l-phosphate; hexosediphosphate; adenosinetriphosphate; yeast nucleate; thymonucleate; phosphoryl choline and lecithin; octanoyl-phosphate; and p-chloranilidophosphonate. The concentrations of substrates ranged from 0.02 to 0.005*M* or, in the case of nucleates and lecithin, from 0.1 to 0.5%. Experiments were performed at pH5, pH7 and pH9. The cation used to trap the phosphate ions was Ca at pH9 and Pb at pH5 and 7. Pyrophosphate gave no positive reaction for enzymatic hydrolysis, and metaphosphate reacted only on the surface of intestinal villi at pH9. Some nonspecific staining was observed, but when this was deleted, the distribution of enzyme was the same for all substrates. The only exception was p-chloranilidophosphonate which gave a different distribution of enxyme in the acid range. Among the other 18 substrates which gave positive reactions, there was no evidence of phosphatases other than the common nonspecific acid and alkaline variety, and the pattern of distribution of enzymatic activity in any given organ was constant and independent of the substrate used.—*H. A. Davenport.*

MEYER, R. L. **A new chromosome stain and its relationship to atypical cell proliferation.** *Proc. Soc. Exp. Biol. and Med.*, **68**, 664–8. 1948.

Salivary glands of *Drosophila* were used as test material. The stain was prepared by dissolving 0.2 g. paraphenylene diamine in 20 ml. of hot 20% acetic acid, adding 0.1 ml. of 30% H_2O_2, and cooling the mixture as soon as a light brown color developed. Oxidation was allowed to continue for about 30 min. before use. To make the stained preparation, isolated glands were suspended on a slide with a drop of 50% acetic acid (they may be pulped to facilitate staining). Contamination with iron from dissecting needles should be avoided. The slides were stained as follows: Blot off the acetic acid and apply the staining solution. Allow to remain in a covered Petri dish over moist filter paper for 1 hr. Apply a cover glass and make squash preparation in the usual way. A second method suggested was to dissolve 10 mg. of quinone diimine crystals in 1 ml. of 70% acetic acid and apply to the material to be stained. The stain develops in 10–20 min. Chromosomes become dark purple-brownish while cytoplasm remains almost unstained. The s-dimethyl- and tetramethyl paraphenylenediamine as well as quinone chlorimine and quinone dichlorimine did not stain. Chromosomes stained with quinone diimine resembled those stained with aceto-carmine, aceto-orcein and the Feulgen technic. It is suggested that the formation of the stain is due to the fixation of the staining agent by the tissue as an analido-quinone type of compound, which involves nucleo-protein. Attention is called to the relationship of this reaction to atypical epithelial proliferations and cancer formation which are caused by certain aromatic amines.—*H. A. Davenport.*

INDEX TO VOLUME 24

Aceto-carmine (*See Carmine, aceto*)
Aceto-lacmoid, 96, 97
Aceto-orcein, 95, 96
Acid fuchsin (*See Fuchsin, acid*)
Acid green, 103, 106
Acid phosphatase, 254
  chemical and histochemical study of the technic for, 147
Acid thionin stain for Nissl bodies on frozen sections, 143
Acridine orange, 70, 71, 253
Acriflavin, 72
Alizarin red S, comparison with a recent German sample, 8
Alkaline phosphatase and glycogen, demonstrating the presence of in the same section, 215
American stains, comparison of with recent German products, 1
Amphibian yolk granules, differential staining of, 249
Angier, J. (*See Levaditi, J. C.*)
Aniline blue, W. S., 229, 230, 232
  comparison with a recent German sample, 4
Animal microtechnic (*Abs. section*), 68, 133, 196, 251
Animal tissue, embedding of, 87
  staining of, 73, 103, 127, 197, 198, 255
Anterior hypophysis, rat, an azocarmine stain for differential cell analysis of, 103
Arcadi, J. A. Hortega's silver carbonate method applied to gill parasites; with a note on the head organs, (abs.), 251
Argentaffin cells, a five-hour variant of Gomori's methenamine silver method for, 225
Arzac, J. P. and Flores, L. G. The histochemical demonstration of glycogen by silver complexes, 25
Auramine O, 70
  comparison with a recent German sample, 8
Azocarmine B, comparison with a recent German sample, 3, 5
Azocarmine G, 103–107
  comparison with a recent German sample, 3, 5
Azocarmine stain for differential cell analysis of the rat anterior hypophysis, 103
Azure A, 72, 207, 211, 213
Azure B, 208, 211, 213
Azure C, 207, 213

Bacteria, cytology of, 199
  staining of, 71, 165, 252
Bacterial cells, combination with certain fluorane derivative dyes at different hydrogen ion concentrations, 217
Baker, H. and Bloom, W. L. Further studies on the Gram stain, 198
Baker, R. F. (*See Hillier, J.; Pease, D. C.*)
Banny, Theresa M. and Clark, George. A simplified method for the double embedding of tissue, 223
Barnes, J. M. The staining of the duodenal mucosa of rats following the injection of solutions of tannic acid, (abs.), 196
Bartholomew, James W. (*See Evans, E. Edward*)
Basic fuchsin (*See Fuchsin, basic*)
Bayliss, M., Glick, D. and Siem, R. A. Demonstration of phosphatases and lipases in bacteria and true fungi by staining methods and the effect of penicillin on phosphatase activity, (abs.), 199
Biebrich scarlet, 191
Biological Stain Commission, membership list, 1949, 193
Bishop, Charles J. Pollen tube culture on a lactose medium, 9
Bismarck brown Y, 72, 85, 168, 231, 232, 233, 234, 235
Black, L. A. (*See Levine, B. S.*)
Block stain, Feulgen-picric-acid, additional data, 127
Blood, microtechnic of, 67, 69, 70, 133, 134, 251, 254
Blood pigments, red, selective demonstration of, 13
Bloom, W. L. (*See Baker, H.*)
Bowden, Wray M. Some modifications of Warmke's permanent section-smear method for plant chromosomes, 171
Boyd, George A. and Williams, Agnes I. Stripping film technics for histological autoradiographs, 254
Branch, G. E. K. and Tolbert, B. M. The spectra of some diaminotriphenylmethane dyes in concentrated sulfuric acid, (abs.), 196
Brecher, George. The structure of unstained reticulocytes, (abs.), 251
Brilliant cresyl blue, 39–45, 72, 251
  as a stain for plant chromosomes, 33
Brilliant green, 233
  comparison with German sample, 7
Briseno-Castrejon, Benjamin and Finerty, John C. An azocarmine stain for differential cell analysis of the rat anterior hypophysis, 103
Broadfoot, H. H. and Schwarz, E. R. An improved permanent mounting medium for textile fibers, (abs.), 252

Brode, W. R. (*See Morris, R. J.*)
Bruce-Mitford, Margaret (*See Jaques, Louis B.*)
Bucher, Gordon E. The anatomy of *Monodontomerus dentipes* Boh., an entomophagous chalcid, (abs.), 197
Bunting, Henry. The histochemical detection of iron in tissues, 109
Burtner, H. Jacquier and Lillie, R. D. A five-hour variant of Gomori's methenamine silver method for argentaffin cells, 225
Burton, C. J. Electron microscopy, (abs.), 195

Carbon fuchsin (*See Fuchsin, carbol*)
Carmalum, 202
Carmine, 69, 80, 198, 215, 216
  aceto, 11, 39, 40, 45, 47, 51, 52, 96, 177
  propiono, 47, 51, 52
Carrera, G. M. and Changus, G. W. Demonstration of acid phosphatase in *Endamoeba histolytica*, (abs.), 254
Carter, George H. (*See Laecom, Rodney C.*)
Castle, William B. (*See Daland, Geneva A.*)
Cell inclusions, hematoxylin-neutral-red as a stain for, 73
Celloidin-rosin-paraffin method, rapid, for embedding tissues, 87
Celloidin sections, thick, of nerve tissue, plastic embedding of, 137
Changus, G. W. (*See Carrera, G. M.*)
Chemical and histochemical study of the technic for acid phosphatase, 147
China blue, 80
Choline esterase activity, 255
Christensen, W. Blake. Observations on the staining of *Corynebacterium diphtheriae*, 165
Chromosomes, plant, brilliant cresyl blue as stain for, 39
Chromosomes, root tip, hydrochloric acid as a fixative for, 95
Chromosome smear preparations; Sudan black B, a new stain for, 177
Chromotrope 2R, 191
Clark, George (*See Banny, Theresa M.*)
Clayson, D. H. F. and Pirie, D. G. Observations on the proposed grading of ice cream by a methylene blue test, (abs.), 196
Clyman, Martin (*See Davidson, Harold B.*)
Cohen, Isadore. Sudan black B—A new stain for chromosome smear preparations, 177
Cole, V. W. Polarized light in photomicroscopy, (abs.), 67
Combination of certain fluorane derivative dyes with bacterial cells at different hydrogen ion concentrations, 217
Comer, J. J. and Hamm, F. A. Modified silica replica technique, (abs.), 195
Comparison of American stains with recent German products, 1
Concentrated formalin versus a 10% solution as a fixative preceding silver staining, 25
Congo red, 80, 232
  comparison with a recent German sample, 3, 6
Conn, H. J. (*See Darrow, Mary A.*)
Cook, A. H., Elvidge, J. A. and Heilbron, Sir Ian. Fertilization, including chemotactic phenomena in the Fucaceae, (abs.), 199
Corynebacterium diphtheriae, observations on the staining of, 165
Cotton blue, 71, 80, 199
Cottrell, Helen J. Tetrazolium salt as a seed germination indicator, (abs.), 198
Crabb, Edward D. A rapid rosin-celloidin-paraffin method for embedding tissues, 87
Crossmon, Germain C. The "dispersion staining" method for the selective coloration of tissue, 61
  The counting of red blood cells by darkfield illumination, (abs.), 67
  Mounting media for phase microscope specimens, 241
Cruickshank, J. C. A simple method for testing dye sensitivity of *Brucella* species, (abs.), 199
Crystal violet, 85, 86, 171, 172, 173, 175, 176, 233, 253
  comparison with a recent German sample, 5
Cytochemistry, 135, 136
Cytological staining, 39, 47, 68, 70, 95, 171, 177, 256

Dacanay, Joaquin G. A method for mass preparation of multiple histopathological slides, 99
Daland, Geneva A. and Castle, William B. A simple and rapid method for demonstrating sickling of the red blood cells: the use of reducing agents (abs.), 133
Darkfield microscopy, 67
Darrow, Mary A. Staining the tubercle organism in sputum smears, 93
Darrow, Mary A., Knapp, Frances, Stotz, Elmer and Conn, H. J. A comparison of American stains with recent German products, 1
Dass, C. M. S. Whole mounts of flat and round worms for morphological studies, 229
Davenport, H. A. (*See Heller, Richard E.; Lhotka, John F.; Porter, Robert W.*)

Davidson, Harold B., Clyman, Martin and Winston, Ruth L. A new fixative for vaginal smears, 145
Davies, Helen and Harman, Pinckney J. A suggestion for prevention of loose sections in the Bodian protargol method, 249
Davis, H. L. and Rynkiewicz, H. J. A fiber identification stain, (abs.), 68
Delafield's hematoxylin combined with safranin for anatomical preparations of plant parts, 191
DeLamater, Edward D. Basic fuchsin as a nuclear stain for fungi, (abs.), 134
Demonstrating the presence of alkaline phosphatase and glycogen in the same section, 215
Demonstration, histochemical, of glycogen by silver complexes, 25
Demonstration of a new nerve-cell organoid, 201
Deterioration of Schiff's reagent, 237
Dichromate-silver method, Golgi's, 117
Differential cell analysis of the rat anterior hypophysis, an azocarmine stain for, 103
Differential staining of amphibian yolk granules, 249
Differentiating yeast ascospores and vegetative cells, 85
"Dispersion staining" method for the selective coloration of tissue, 61
Double embedding of tissue, simplified method for, 223
Dowding, Eleanor Silver. The spores of *Histoplasma*, (abs.), 199
Doyle, William L. Effects of dehydrating agents on phosphatases in the lymphatic nodules of the rabbit appendix, (abs.), 254
Duffett, R. E. Hematoxylin-neutral red. A stain for cell inclusions and certain tissue elements, 73
Dyes and their biological uses (*Abs. section*), 133, 196, 251

Electron microscope, 67, 134, 195, 251
Elvidge, J. A. (*See Cook, A. H.*)
Embedding, 87, 117
  double, simplified method for, 223
  plastic, of thick celloidin sections of nerve tissue, 137
Engström, A. Quantitative cytochemical determination of nitrogen by X-ray absorption spectrography, (abs.), 134
  Quantitative micro- and histochemical elementary analysis by Roentgen absorption spectrography, (abs.), 135
  Ultramicroanalysis by X-ray absorption spectrography, (abs.), 135
Engström, A. and Jakus, M. A. Intracellular determination of protein by X-ray microspectrography, (abs.), 135
Engstrom A. and Lindström, B. Histochemical analysis by X-rays of long wavelengths, (abs.), 135
Eosin B, 218, 220, 221
Eosin Y, 69, 80, 136, 191, 197, 218, 220, 221, 250
  comparison with a recent German sample, 7
Erythrocruorin, selective demonstration of, 13
Erythrosin, 191, 197
  comparison with a recent German sample, 6
Erythrosin B, 218, 220, 221, 233
Evans, E. Edward, King, Carl W. and Bartholomew, James W. Differentiating yeast ascospores and vegetative cells, 85
Evans, Richard I. Delafield's hematoxylin combined with safranin for anatomical preparations of plant parts, 191

Fajer, A. (*See Rabinovitch, M.*)
Fast green FCF, 231, 232, 234, 235
Feulgen stain, 19, 237, 253
Feulgen-picric-acid block stain, additional data, 127
Fiber identification, 68, 134
Figge, Frank H. J., Weiland, G. S. and Manganiello, L. O. J. Cancer detection and therapy. Affinity of neoplastic, embryonic, and traumatized tissues for porphyrins and metalloporphyrins, (abs.), 251
Finerty, John C. (*See Briseno-Castrejon, Benjamin*)
Five-hour variant of Gomori's methenamine silver method for argentaffin cells, 225
Fixative for vaginal smears, 145
Fixing agents, 33, 95
Flat worms, whole mounts of for morphological studies, 229
Flint, Thomas J. and Matzke, Edwin B. Recent procedures for the study of three-dimensional shapes of resting cells, and a new method for the shape study of cells in division, (abs.), 70
Flores, L. G. (*See Arzac, J. P.*)
Fluorane derivative dyes, combination with bacterial cells at different hydrogen ion concentrations, 217
Fluorescein, 252
Fluorescence microscopy, 70, 71, 195, 197
Formalin, concentrated, versus a 10% solution as a fixative preceding silver staining, 25
Friend, James R., Jr. (*See Rosenberg, Lauren E.*)

Frozen sections, acid thionin stain for Nissl bodies on, 143
Fuchsin, acid, 13, 14, 16, 68, 71, 88, 191, 197, 230, 233
comparison with a recent German sample, 3, 6
Fuchsin, basic, 21, 22, 28, 70, 72, 85, 93, 134, 171, 172, 173, 199, 237, 253
Fuchsin, carbol, 85, 93, 94, 252, 254
Fuchsin, ponceau, as a routine counterstain, 191
Fungi, staining of, 71, 134, 199, 254
Furman, N. H. and Stone, K. G. A polarographic study of certain anthraquinones, (abs.), 133

Gentian violet, 85, 198
comparison with a recent German sample, 3, 5
German stains, recent, comparison of with American products, 1
Gerstel, D. U. Hydrochloric acid as a fixative for root tip chromosomes, 95
Getty, R. (*See Merrill, R. C.*)
Giemsa stain, 254
Glick, D. (*See Bayliss, M.*)
Glycogen, histochemical demonstration of by silver complexes, 25
staining of, 198
Glycogen and alkaline phosphatase, demonstrating the presence of in the same section, 215
Goldman, Morris. A single solution iron-hematoxylin stain for intestinal protozoa, 57
Golgi's dichromate-silver method. 1. Effects of embedding. 2. Experiments with modifications, 117
Gomori, G. Histochemical demonstration of sites of choline esterase activity, (abs.), 255
Histochemical demonstration of sites of phosphamidase activity, (abs.), 255
Histochemical specificity of phosphatases, (abs.), 256
Gomori's methenamine silver method for argentaffin cells, a five-hour variant of, 225
Gram stain, 198, 200, 253
Griggs, Frederick C. The chromosome smear technique: A critical review and improvement of method, (abs.), 68
Groat, Richard A. Initial and persisting staining power of solutions of iron-hematoxylin lake, 157

Hamm, F. A. (*See Comer, J. J.*)
Harman, Pinckney J. (*See Davies, Helen*)
Harris, J. O. Combination of certain fluorane derivative dyes with bacterial cells at different hydrogen ion concentrations, 217
Hayes, E. Russell. A rigorous redefinition of the plasmal reaction, 19
Heilbron, Sir Ian (*See Cook, A. H.*)
Heller, Richard E., Starr, Grier F. and Davenport, H. A. Thiazin dyes in supravital staining of nerve fibers, 207
Hematein, 158
Hematoxylin, 13, 57–60, 69, 73–77,100, 101, 103, 104, 105, 109, 110, 112, 113, 114, 136, 141, 157–163, 191, 192, 197, 232, 233, 235, 253
Hematoxylin, iron lake, initial and persisting staining power of, 157
Hematoxylin-neutral-red. A stain for cell inclusions and certain tissue elements, 73
Hemoglobin, selective demonstration of, 13

Heparin, metachromatic activity of, 72
Hillier, J. Some remarks on the image contrast in electron microscopy and the two-component objective, (abs.), 251
Hillier, J., Knaysi, G. and Baker, R. F. New preparation techniques for the electron microscopy of bacteria, (abs.), 195
Hirschler, Jan. The selective demonstration of red blood pigments (hemoglobin and erythrocurorin) in microscopic balsam preparations, 13
Histochemical demonstration of glycogen by silver complexes, 25
Histochemical detection of iron in tissues, 109
Histochemistry, 147, 199, 215, 253
Histochemistry (*Abs. section*), 72, 134, 200, 254
Histopathological slides, multiple, a method for mass preparation of, 99
Hitier, H. and Quidet, P. Obtention de plantes polyploides par traitement a l'hexachlorocyclohexane et au sulfure de polychlorocyclane, (abs.), 70
Hock, C. W. (*See Kingsinger, W. G.*)
Hsu, J. H. An improved method for counting blood platelets, (abs.), 69
Hunter, Lillian M. A study of the mycelium and haustoria of the rusts of *Abies*, (abs.), 71
Hydrochloric acid as a fixative for root tip chromosomes, 95

Illumination for microscope, 195
Immersion oils, 196
Indicators, dyes as, 196, 198, 199, 253
Indigo carmine, comparison with a recent German sample, 3, 5
Indulin, 80

Initial and persisting staining power of solutions of iron-hematoxylin lake, 157
Insects, microtechnic for, 69, 197, 252
Intestinal protozoa, a single solution iron-hematoxylin stain for, 57
Invertebrate histology, 229
Iodine green, 233
Iron-hematoxylin lake, initial and persisting staining power of, 157
Iron-hematoxylin stain, single solution, for intestinal protozoa, 57
Iron in tissues, histochemical detection of, 109
Isenberg, H. D. Simplified method for staining spermatozoa, (abs.), 252

Jakus, M. A. (*See Engström, A.*)
Janus green, 76
Jaques, Louis B., Bruce-Mitford, Margaret and Ricker, Ann G. The metachromatic activity of heparin, (abs.), 72
Jelley, Edwin E. Light microscopy, (abs.), 195
Junqueria, L. C. (*See Rabinovitch, M.*)

King, Carl W. (*See Evans, E. Edward*)
Kinsinger, W. G. and Hock, C. W. Electron microscopical studies of natural cellulose fibers, (abs.), 134
Kitzmiller, J. B. The use of dioxane in insect microtechnieque, (abs.), 252
Knapp, Frances (*See Darrow, Mary A.*)
Knaysi, G. Preliminary observations on germination of the spores of *Bacillus mycoides* in a nitrogen-free medium and certain properties of the transparent cells, (abs.), 200
Knaysi, G. (*See Hillier, J.*)
Kramer, Sol. A staining procedure for the study of insect musculature, (abs.), 69
Kugler, J. H. Ponceau-fuchsin as a routine counterstain, 191

Laboratory hints from the literature, 67–72, 133–136, 195–200, 251–257
Lactose medium, pollen tube culture on, 9
Laecom, Rodney C. and Carter, George H. Erythrocytes in urinary sediment: identification and normal limits, with a note on the nature of granular casts, (abs.), 69
Laskey, Alice M. Acid thionin stain for Nissl bodies on frozen sections, 143
Lassek, A. M. and Powers, Margaret M. Concentrated formalin versus a 10% solution as a fixative preceding silver staining, 25
Laufer, Ira J. The differential staining of amphibian yolk granules, 249
Leucofuchsin, 130
Levaditi, J. C., Lepine, P. and Angier, J. Etude au moyen de la microscopie en fluorescence, des includions intro-cellulaires acidophiles provoquées par les virus de la rage, de la vaccine, de la maladie de Borne, (abs.), 197
Levine, B. S. and Black, L. A. Newly proposed staining formulas for the direct microscopic examination of milk, (abs.), 71
Levine, P. (*See Levaditi, J. C.*)
Lhotka, John F. and Davenport, H. A. Deterioration of Schiff's reagent, 237
The Feulgen-picric-acid block stain. Additional data, 127
Light green SF yellowish, 71, 76, 233
comparison with a recent German sample, 7
Lillie, R. D. (*See Burtner, H. Jacquier*)
Lindström, B. (*See Engström, A.*)
Livingston, R. and Pariser, R. The chlorophyll-sensitized photo-oxidation of phenylhydrazine by methyl red. II. Reactivity of the several forms of methyl red, (abs.), 72
Lord, Thomas H. and Smull, Margaret M. An improved microscopic method of examining fatty foods, (abs.), 252

Malachite green, 85, 165–168, 196
comparison with a recent German sample, 7
Manganiello, L. O. J. (*See Figge, Frank H. J.*)
Markell, E. K. (*See Michael, Paul*)
Marshall, A. H. E. A method for the demonstration of reticulo-endothelial cells in paraffin sections, (abs.), 197
Martin, B. F. A method for demonstrating the presence of alkaline phosphatase and glycogen in the same section, 215
Mass preparation of multiple histopathological slides, a method for, 99
Mass production of permanent smear preparations, treatment of *Trillium erectum* prior to and during, 47
Mast cells in mouse skin, rapid technic for demonstrating, 185
Matzke, Edwin B. (*See Flint, Thomas J.*)
May, J. Zur fluoreszenzmikroskopischen Unterscheidung lebender und toter Bakterien mittels Akirdinorangefärbung, (abs.), 253
Mayhew, R. L. A method for using methylene blue and eosin as a stain for sections, (abs.), 197
Membership list, 1949, Biological Stain Commission, 193
Merrill, R. C., Spencer, R. W. and Getty, R. The effect of sodium silicates on

the absorption spectra of some dyes, (abs.), 133
Methenamine silver method for argentaffin cells, Gomori's, a five-hour variant of, 225
Methyl green, 22, 136, 165, 166
Methyl orange, comparison with a recent German sample, 8
Methyl red, 72
Methyl violet, 199
comparison with a recent German sample, 5
Methylene blue, 71, 85, 94, 165, 166, 168, 185, 186, 188, 196, 197, 200, 207, 208, 209, 210, 211, 213, 232, 233, 252, 253
comparison with a recent German sample, 4
Methylene green, 207, 212, 213, 251
Methylene violet, 208, 213
Meyer, R. L. A new chromosome stain and its relationship to atypical cell proliferation, (abs.), 256
Michael, Paul and Markell, E. K. Modified Papanicolaou stain for amebae, (abs.), 253
Microörganisms (*Abs. section*), 71, 134, 199, 252
Microradiography, 134, 135, 254
Microscope and other apparatus (*Abs. section*), 67, 195, 251
Microtechnic in general (*Abs. section*), 68, 195
Miller, E. C. (*See Price, J. M.*)
Miller, J. A. (*See Price, J. M.*)
Morley, Thomas. Staining of plant materials cleared in NaOH, 231
Morris, R. J. and Brode, W. R. The relation between the absorption spectra and the chemical composition of dyes. XX. Induced noncoplanarity in symmetrical benzidine dyes, (abs.), 133
Morrison, P. H. (*See Singer, M.*)
Mounting media, 252
for phase microscope specimens, 241
Mouse skin, rapid technic for demonstrating mast cells in, 185
Multiple histopathological slides, a method for mass preparation of, 99

Negative-staining of protozoa with nigrosin, 79
Nerve-cell organoid, new, demonstration of, 201
Nerve fibers, supravital staining with thiazin dyes, 207
Nerve tissue, plastic embedding of thick celloidin sections of, 137
Nervous tissue, impregnation of, 198
Neutral red, 72, 73–77, 200
comparison with a recent German sample, 6
Neutral violet, 76
New methylene blue, 207, 212, 213, 251
Nichols, J. Improved method for handling photolabile liquids, (abs.), 68
Nichols, M. Starr. Gentian (crystal) violet solution for Gram stain, (abs.), 253
Nigrosin, 79–84
negative staining of protozoa with, 79
Nile blue, 72
comparison with a recent German sample, 4
Nissl bodies on frozen sections, acid thionin stain for, 143
Notes on technic, 191–192, 249–250
Nowak, A., Jr. (*See Zahl, Paul A.*)

Observations on the staining of *Corynebacterium diphtheriae*, 165
Orange G, 70, 80, 103, 106, 200
comparison with a recent German sample, 7
Organoid, nerve-cell, new, demonstration of, 201

Packard, C. E. The effects of certain chemicals on the macronucleus of *Spirostomum teres*, with notes on the genus, (abs.), 251
Paracarmine, 112, 114
Paraffin-rosin-celloidin method, rapid, for embedding tissues, 87
Pararosanilin, comparison with a recent German sample, 6
Pariser, R. (*See Livingston, R.*)
p-dimethylaminoazobenzene, 200
Pease, D. C. and Baker, R. F. Sectioning techniques for electron microscopy using a conventional microtome, (abs.), 67
Pennington, Derrol. The use of periodate in microbiological staining, (abs.), 253
Phase microscope specimens, mounting media for, 241
Phase microscopy, 67, 68, 195
Phenylhydrazine, photo-oxidation of, 72
Phloxine, 73, 101
comparison with a recent German sample, 3, 6
Phosphatase, dehydration of, 254
specificity of, 255, 256,
Phosphatase, acid, chemical and histochemical study of the technic for, 147
Photolabile liquids, handling of, 68
Photomicrography, 67, 195
Picric acid, 68, 76, 82, 127–131, 192
Picric-acid-Feulgen block stain, additional data, 127
Pigments, red, blood, selective demonstration of, 13
Pirie, D. G. (*See Clayson, D. H. F.*)
Plant chromosomes, brilliant cresyl blue as a stain for, 33
some modifications of Warmke's per-

manent section-smear method for, 171
Plant materials cleared in NaOH, staining of, 231
Plant microtechnic (*Abs. section*), 70, 134, 198, 252
Plant parts, Delafield's hematoxylin and safranin for, 191
Plant tissue, staining of, 191
Plastic embedding of thick celloidin sections of nerve tissue, 137
Poirer's blue, 80
Polarographic analysis, 133
Pollen tube culture on a lactose medium, 9
Ponceau de xylidine, 191
Ponceau-fuchsin as a routine counterstain, 191
Porter, Robert W. and Davenport, H. A. Golgi's dichromate-silver method. 1. Effects of embedding. 2. Experiments with modifications, 117
Powers, Margaret M. (*See Lassek, A. M.*)
Prevention of loose sections in the Bodian protargol method, 249
Price, J. M., Miller, E. C. and Miller, J.A. The intracellular distribution of protein, nucleic acids, riboflavin, and protein-bound aminoazo dye in the livers of rats fed p-dimethylaminoazobenzene, (abs.), 200
Protargol method, Bodian, prevention of loose sections in, 249
Protozoa, intestinal, a single solution iron-hematoxylin stain for, 57
Protozoa, microtechnic for, 253, 254
negative staining of with nigrosin, 79
Prussian blue, 109–114
Pyronin, 72, 136, 196, 199
Pyronin B, comparison with a recent German sample, 3, 7
Pyronin Y (G), comparison with a recent German sample, 3, 7

Queiroz, Lopes A. (*See Serra, J. A.*)
Quidet, P. (*See Hitier, H.*)

Rabinovitch, M., Junqueria, L. C. and Fajer, A. A chemical and histochemical study of the technic for acid phosphatase, 147
Ramsbottom, J. M. and Strandine, E. J. Comparative tenderness and identification of muscles in wholesale beef cuts, (abs.), 198
Rapid rosin-celloidin-paraffin method for embedding tissues, 87
Rapid technic for demonstrating mast cells in mouse skin, 185
Rat anterior hypophysis, an azocarmine stain for differential cell analysis of, 103
Rawson, Arnold J. Acidfast property of *Histoplasma capsulatum*, (abs.), 254
Rhodamine, 252
Rhodamine 6G, 133
Richards, Oscar W. Phase microscopy, (abs.), 67
Ricker, Ann G. (*See Jaques, Louis B.*)
Rigorous re-definition of the plasmal reaction, 19
Root tip chromosomes, hydrochloric acid as a fixative for, 95
Rosanilin, comparison with a recent German sample, 3, 6
Rose bengal, 218, 220, 221
comparison with a recent German sample, 3, 8
Rosenbaum, Robert M. Negative-staining of protozoa with nigrosin, 79
Rosenberg, Lauren E. and Friend, James R., Jr. Plastic embedding of thick celloidin sections of nerve tissue, 137
Rosin-celloidin-paraffin method, rapid, for embedding tissues, 87
Round worms, whole mounts of for morphological studies, 229
Ruthenium red, 70
Rynkiewicz, H. J. (*See Davis, H. L.*)

Safranin O, 85, 86, 168, 169, 191, 192, 231, 232, 233, 251
comparison with a recent German sample, 3, 7
Schertiger, Ann M. (*See Stewart, Wilson N.*)
Schiff's reagent, deterioration of, 237
Schwarz, E. R. (*See Broadfoot, H. H.*)
Sectioning, 67
Seed germination indicator, 198
Selective coloration of tissue, "dispersion staining" method for, 61
Selective demonstration of red blood pigments (hemoglobin and erythrocruorin) in microscopic balsam preparations, 13
Serra, J. A. and Queiroz-Lopes, A. Direct demonstration of basic protein in the chromosome and nucleolus, (abs.), 135
Donnés pour une cytophysiologie du nucléole. L'activité nucléolaire pendant la croissance de l'oocyte chez des Helicidae, (abs.), 136
Une méthode pour la démonstration histochimique du phosphore des acides nucléiques, (abs.), 136
Shropshire, R. F. Oblique photographic illuminator, (abs.), 195
Siem, R. A. (*See Bayliss, M.*)
Silica replication, 195
Silver complexes for histochemical demonstration of glycogen, 25
Silver impregnation, 117, 197, 225, 251
Silver staining, concentrated formalin versus a 10% solution as a fixative preceding, 25

Simplified method for the double embedding of tissue, 223
Singer, M. and Morrison, P. H. The influence of pH, dye, and salt concentration on the dye binding of unmodified and modified fibrin, (abs.), 200
Single solution iron-hematoxylin stain for intestinal protozoa, 57
Smear preparations, permanent, treatment of *Trillium erectum* prior to and during mass production of, 47
Smull, Margaret M. (*See Lord, Thomas H.*)
Soluble blue 2B extra, 68
Some modifications of Warmke's permanent section-smear method for plant chromosomes, 171
Sparrow, A. H. and Sparrow, Rhoda C. Treatment of *Trillium erectum* prior to and during mass production of permanent smear preparations, 39
Sparrow, Rhoda C. (*See Sparrow, A. H.*)
Spectrophotometry, 133, 196
Spencer, R. W. (*See Merrill, R. C.*)
Spermatozoa, microscopic study of, 252
Spirochaetes, staining of, 70, 254
Sputum smears, staining of tubercle organism in, 93
Staining of plant materials cleared in NaOH, 231
Staining the tubercle organism in sputum smears, 93
Starr, Grier F. (*See Heller, Richard E.*)
Stewart, Wilson N. and Schertiger, Ann M. Brilliant cresyl blue as a stain for plant chromosomes, 33
Stone, K. G. (*See Furman, N. H.*)
Stotz, Elmer. (*See Darrow, Mary A.*)
Strandine, E. J. (*See Ramsbottom, J. M.*)
Strugger, S. Fluorescence microscope examination of bacteria in soil, (abs.), 71
Fluorescence microscope examination of trypanosomes in blood, (abs.) 70
Sudan black B, 177–183, 201, 204
a new stain for chromosome smear preparations, 177
Sudan III, 91, 198
comparison with a recent German sample, 3, 8
Supravital staining of nerve fibers with thiazin dyes, 207

Tartrazine, 73
Taylor, E. Wilfred. The practical application of phase-contrast to the biologist's microscope, (abs.), 68
Tetrazolium salt, 198
Thiazin dyes in supravital staining of nerve fibers, 207
Thioflavine S, 197
Thionin, 85, 143, 144, 199, 213, 249, 250
Thionol, 207, 211, 213
Thomas, Owen Lewis. The demonstration of a new nerve-cell organoid, 201
Tissue. (*See Animal tissue or Plant tissue*)
Tolbert, B. M. (*See Branch, G. E. K.*)
Toluidine blue O, 133, 165, 167, 168, 169, 207, 212, 213
comparison with a recent German sample, 3, 4
Toluylene red, 76
Tompkins, Edna H. M. Methods to increase accuracy in the use of Hayem's solution for red blood counts, (abs.), 134
Treatment of *Trillium erectum* prior to and during mass production of permanent smear preparations, 39
Tubercle organism in sputum smears, staining of, 93
Tumor tissue, staining of, 251

Vaginal smears, a new fixative for, 145
staining of, 195, 253
Valade, Paul. Récherches histochimiques sur les lipides de l' écorce cerebrale, (abs.), 198
Vallance-Owen, J. The histological demonstration of glycogen in necropsy material, (abs.), 198
Van Duijn, C., Jr. Substitutes for cedarwood oil, (abs.), 196
Victoria blue, 196
Vital staining, 196, 251

Warmke's permanent section-smear method for plant chromosomes, some modifications of, 171
Weiland, G. S. (*See Figge, Frank H. J.*)
Weinrebe, N. Stain for *Borrelia recurrentis*, (abs.), 254
Whole mounts of flat and round worms for morphological studies, 229
Williams, Agnes I. (*See Boyd, George A.*)
Winston, Ruth L. (*See Davidson, Harold B.*)

Yeast ascospores and vegetative cells, differentiation of, 85
Yolk granules, amphibian, differential staining of, 249

Zahl, Paul A. and Nowak, A., Jr. A rapid technic for demonstrating mast cells in mouse skin, 185